Microbial associations developing on modified atmosphere packaged beef steaks

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MICROBIAL ASSOCIATIONS DEVELOPING ON MODIFIED ATMOSPHERE PACKAGED BEEF STEAKS

Submitted by:

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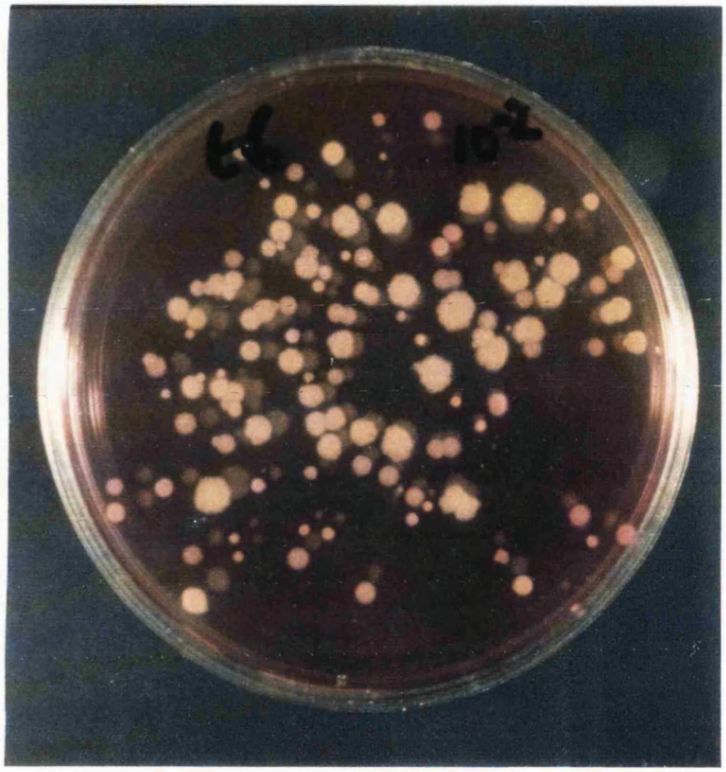

MODIFIED CEPHALORIDINE-FUCIDIN-CETRIMIDE MEDIUM: A SELECTIVE DIFFERENTIAL MEDIUM FOR THE ENUMERATION OF PSEUDOMONAS *SPP.*

Pink colonies should identifiy with *Pseudomonas* spp. and yellow ones with Enterobacteriaceae. For further details see Chapter 2.

ABSTRACT

Modified atmosphere packaging (MAP) of meats has increased in commercial importance over the past 20 years. The ecosystems of such packs leads to development of different microbial populations *vis-à-vis* those with aerobic storage. These populations have been analysed previously using selective media to enumerate particular microbial groups, but the succession of species developing in such ecosystems has not been determined. This was done in the present study of beef steaks packed under vacuum or in 50% N_2 + 50% CO_2, 80% O_2 + 20% CO_2 or 100% CO_2 and stored at 0 or 5 °C.

Many of the selective media chosen to enumerate the microbial groups were found not to be entirely satisfactory during preliminary work. In some instances modifications of an existing medium (e.g. CFC for pseudomonads) aided enumeration. Members of the numerically dominant lactic acid bacteria (LAB) as well as the pseudomonads and Enterobacteriaceae were identified. *Brochothrix thermosphacta* was enumerated on the highly selective Streptomycin Thallous Acetate agar and not identified further.

Carnobacterium divergens and *Lactobacillus sake* tended to predominate the microflora of MAP beef at 5 °C. Leuconostocs were predominant on beef steaks in 100% CO_2. The species of lactic acid bacteria on the MAP beef changed during storage. Their growth was affected by CO_2 and acetic acid. *Pseudomonas fragi* was the most frequently isolated of the pseudomonads, although *Ps. fluorescens* and *Ps. lundensis* were both present on MAP beef. *Hafnia alvei, Serratia liquefaciens* and *Pantoea agglomerans* all formed relatively high proportions of the isolates from Violet Red Bile Glucose agar. The atmosphere and temperature of storage, as well as the composition of the microflora present on the meat at the time of packaging, altered the microbial population.

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CONTENTS

INTRODUCTION

Meat is an important part of the diet of the British people. Indeed, in 1989 £7 billion was spent on red meats in the UK (Anon, 1991). Traditionally, meat was bought from a butcher and packed in greaseproof paper or more recently plastic bags. In the past twenty years, however, there have been changes in the lifestyle of consumers, the marketing stategies of supermarkets and in packaging technologies as well as an increase in fuel prices. The combination of these factors renewed interest in modified atmosphere packaging (MAP), a method used in the 1930's for the shipment of meat from Australasia to the UK (Lawrie, 1991). Modified atmosphere packaging involves the enclosure of a food product in a film with high gas barrier properties. This reduces microbial growth, slows respiration rates and retards enzyme spoilage. The adoption of MAP for red meats gives an extension of shelf-life for beef from 5 days at 5 °C (aerobically stored meats) to 11-13 days in 75% O_2 + 25% CO_2 at 6 °C (Nortjé and Shaw, 1989) or >51 days in 100% CO_2 at 4 °C (Erichsen and Molin, 1981). Although MAP is relatively widely used, there is concern over the safety of MAP meat, particularly with respect to the potential growth of pathogens. In aerobically stored meats pungent odours produced by the major spoilage organisms, pseudomonads, mark the end-point of storage. In contrast, the carbon dioxide used commonly in MAP tends to suppress the growth of these micro-organisms and favours that of lactic acid bacteria. The growth of the latter produces less offensive sour rather than "off" odours.

In order to ascertain the potential for pathogen growth in MAP meat, a Food-Linked Agro-Industrial Research programme was funded by the EEC. The title of the programme was "Improving the safety and quality of meat and meat products and assessment by novel methods". It was a collaborative venture between the Institute for Technology of Agricultural Products, Athens; the National Food Centre, Dublin; Leatherhead Food Research Association, Leatherhead, England, and Bath University. It involved the inoculation of different meats (beef, lamb and chicken) with pathogens (*Aeromonas hydrophila*, verotoxigenic *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurium* and *Yersinia enterocolitica*) and subsequent packaging and storage in typical MAP conditions (vacuum pack, 50% N_2 + 50% CO_2, 80% O_2 + 20% CO_2, 100% CO_2 all stored at 0 or 5 °C). The fate of the pathogens was determined over the storage period.

The other major aspect of the project was a study of the spoilage flora. The work was done at Bath University and aimed to identify the important members of the microbial

associations during storage of beef in MAP. Selective media were used to determine the overall proportions of each bacterial group found in MAP meats, an approach used in many previous studies. In the present study, however, the work was extended. Bacteria were isolated, characterised and identified in order to determine the most important species in the microbial associations. The importance of a species may not mean that it has to be numerically dominant. Spoilage taints and odours may be produced by a minor portion of the flora. Consequently, all bacterial groups found commonly in MAP meats were studied.

CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

Preservation of foods involves reduction in the numbers of micro-organisms or the control of their growth. This may involve low temperature storage, thermal processing, irradiation, control of water activity, control of pH, the addition of preservatives, fermentation etc., often in conjunction with packaging (Sofos, 1993). Traditionally, meat was consumed shortly after slaughter or it was preserved by low temperatures (in polar regions or more recently by refrigeration), drying (by salting, smoking or insolation) or fermentation (Nychas *et al.*, 1988).

Fresh red meat is an important part of the British diet. The expenditure on fresh meat in 1989 was estimated as £3 billion on beef, £3 billion on pork and £1 billion on lamb (Anon., 1991). A general change in the lifestyle of the people of Western Europe has tended to decrease the patronage of local butchers shops, with supermarkets assuming a dominant role. Supermarkets also changed from the traditional packaging of fresh meat, either in greaseproof paper wrapping or plastic bags, to the display of meat on a rigid tray with a covering of a gas-permeable film. This protected the meat surfaces from casual contamination, retained moisture and, most importantly, allowed consumer selection. Initially, purchase decisions were based mainly on meat colour and price. Now a multitude of factors such as health (a desire for fresh/chilled, preservative-free products), convenience, ease of preparation, portion control and shelf-life (Rice, 1990; Farber, 1991) affect the decision.

New polymeric materials have been developed and energy costs have increased (Smith *et al.*, 1990a). These changes together with altered consumer habits have led to an increased interest in the use of modified atmosphere (MAP) and controlled atmosphere packaging (CAP). These systems have been defined by Koski (1988) as:

MAP "Enclosure of food products in high gas barrier materials, in which the gas environment has been changed *once* to slow respiration rates, reduce microbiological growth, and retard enzyme spoilage - with the final effect of lengthening shelf life.". Vacuum packaging (VP) is included in MAP.

CAP "Enclosure of food products in variable gas barrier materials, in which the gaseous environment has been changed and is selectively controlled to increase shelf life".

It has been known for a long time that the shelf life of meat can be extended by an increase in the concentration of carbon dioxide in the storage atmosphere. By the late 1930's most of the beef (60%) and some of the lamb (26%) shipped from Australia and New Zealand to the UK was stored in an atmosphere enriched with carbon dioxide (Lawrie, 1991). It was only during the 1970's, however, that there was renewed interest in the use of modified atmospheres particularly for individual primal joints. In 1979 Marks and Spencers launched a range of gas-flushed packs "ATMOSPAK" of fresh meat products in the UK (Muller, 1986). High standards of hygiene and temperature control are essential prerequisites for the quality and safety of MAP packed meat. Day (1990) drew attention to the common misconception that MAP will overcome poor hygiene; it will only prolong the shelf life of a high quality product. Marks and Spencers critically selected the companies used to produce the "ATMOSPAK" system for meats in order to ensure good levels of hygiene and meat quality (Muller, 1986) as well as improved control along the entire distribution chain (Rice, 1990). Since that time there has been an increase in the commercial exploitation of MAP in the meat industry. This is especially so in Europe where there are short distances between centralised packing operations and shops together with a marked consumer acceptance of such packaging systems. In the UK 35-40% fresh meat is MAP (Brody, 1993). The length of the distribution routes in the USA is such that MAP packs have an insufficient shelf-life (Williams, 1988).

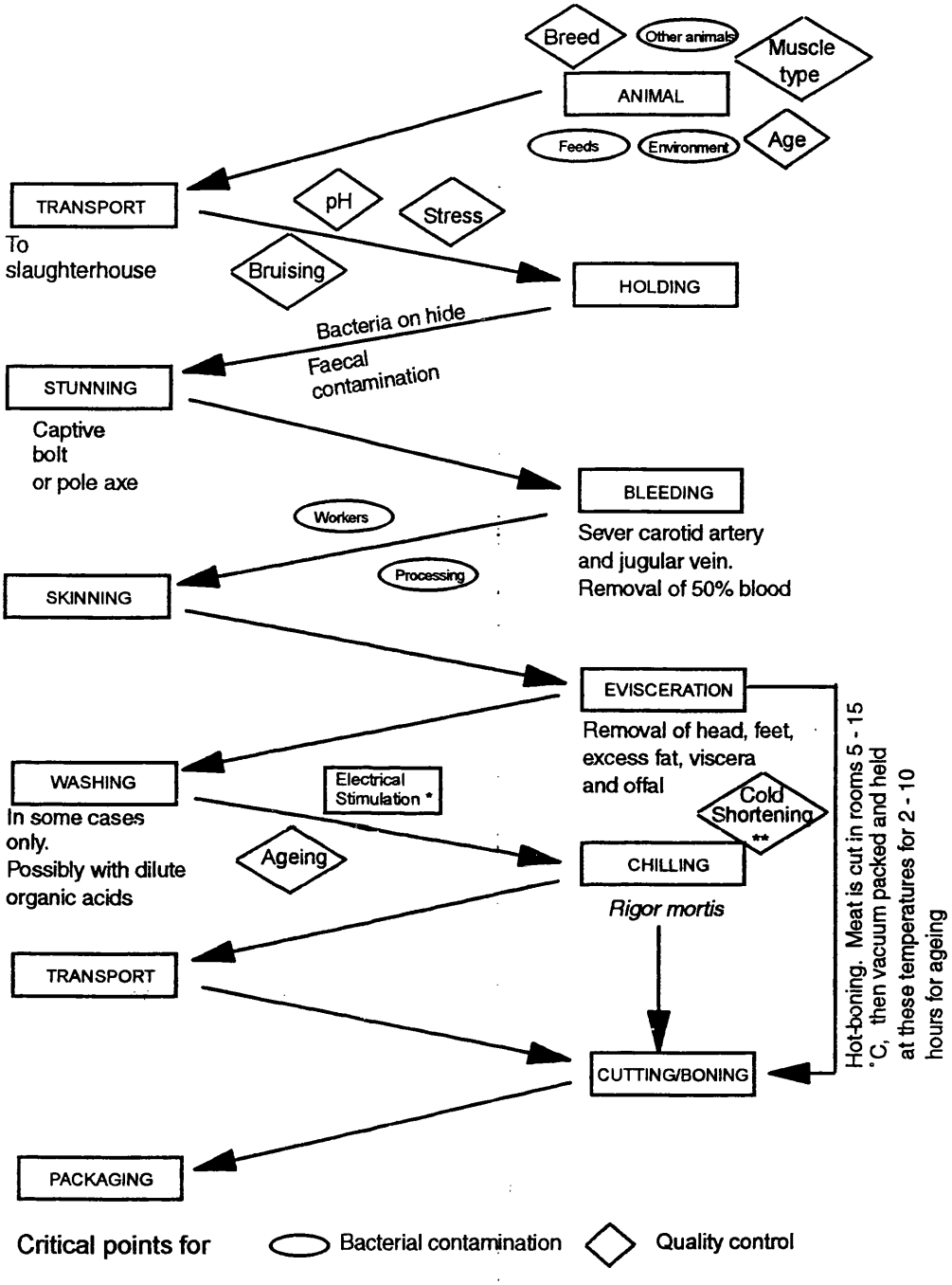
THE ECOLOGY OF FRESH RED MEAT

Formation of meat from muscle tissue

Meat, the flesh of mammals used as food, is formed as a result of a series of reactions *post mortem* (Figure 1.1). Muscle fibres *in vivo* are surrounded by the sarcolemma to which the connective tissue is attached. The fibres consist of myofibrils, composed of actin and myosin microfilaments, with sarcoplasm and sarcoplasmic reticulum (a fine network of tubules) between them (Lawrie, 1991). There are three major muscle fibre types in meat, slow-twitch oxidative, fast-twitch glycolytic and fast-twitch oxidative-glycolytic. The relative proportions of these fibre types determines the overall appearance of muscles (Renerre, 1990).

Glycolysis converts glycogen present in the muscles at slaughter to lactic acid with the production of ATP (Figure 1.2). Creatine phosphate is also converted to creatine with the concomitant production of ATP. These anaerobic processes, however, do not maintain

physiological levels of ATP required for tissue respiration. Once the ATP in the myofibrils has been depleted, actin and myosin combine to form actomyosin - a rigid structure. This is the cause of *rigor mortis*.



* Electrical stimulation (the application of a pulsed AC voltage) is not in general use. The procedure leads to rapid onset of rigor, tenderization of the meat and prevention of toughening.

** Cold shortening occurs if the rate of chilling is too rapid - it leads to tough meat

Figure 1.1 Control points for quality and bacterial contamination during the slaughter of cattle

Adapted from Lawrie (1991) and Anon. (1991)

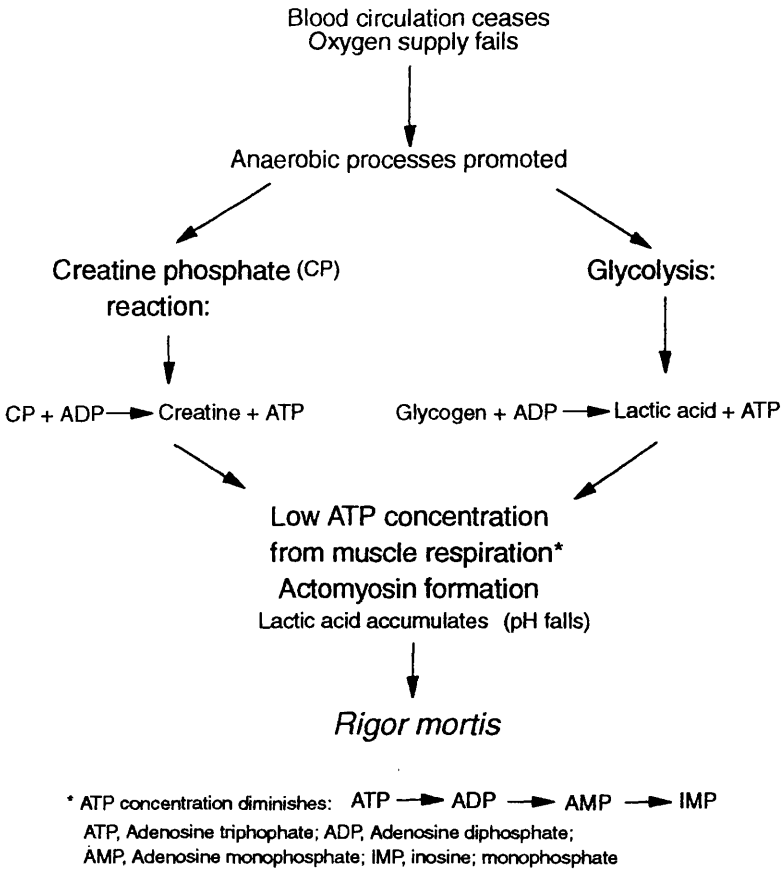


Figure 1.2 The consequences of circulatory failure in muscle tissue
Adapted from Lawrie (1991) and Nychas *et al.* (1988)

The formation of lactic acid during glycolysis causes a drift in muscle pH to the ultimate pH, approximately 5.4 - 5.5. If animals are stressed or exercised prior to slaughter the glycogen stores are depleted and the pH drift is limited. This results in the formation of dry, firm, dark meat (DFD) with an ultimate pH $\geq$ 6.0. The pH (5.4-5.5) of normal meat has important effects on the muscle tissue. For example, some of the sarcoplasmic proteins are denatured such that they are more susceptible to attack by proteases released when the membranes of the lysosomes are perturbed by low pH (Lawrie, 1991).

Muscles become pliable again after *rigor mortis*, but not due to the dissociation of the actomyosin. The subsequent mechanisms leading initially to tenderization during the ageing process following *rigor* have yet to be elucidated. It may be associated with the disruption of the sarcoplasmic network around the myofibrils (Lawrie, 1991). If contracted muscles enter *rigor*, many cross-links are formed in the actomyosin. This leads to tough meat.

Meat as a substrate

Differences due to breed, husbandry and slaughter practice means that the composition of meat is variable. The typical composition of lean meat is shown is Table 1.1. It is obvious that meat is a good medium for microbial growth.

Table 1.1 Typical composition of meat tissue after *rigor mortis*

Component	Wet % weight
Water	75
Protein	19
Fat	2.5
Carbohydrate	1.2
including lactic acid	0.9
glucose-6-phosphate	0.17
glycogen	0.1
glucose	0.01
Nitrogenous soluble substances	1.65
including creatine	0.55
inosine monophosphate	0.3
di- and tri-phosphopyridine nucleotides	0.07
amino acids	0.35
dipeptides (carnosine, anserine)	0.3
Inorganic soluble substances	0.65
Vitamins	Quantitatively minute

Adapted from Lawrie (1991)

Adipose tissue contains 99% fat - esters of glycerol with fatty acids (Lawrie, 1991) which becomes covered with blood and meat exudate during slaughter and processing. Gill and Newton (1980a) found low levels of all the nutrients necessary for microbial growth in such tissue, glucose-6-phosphate being the only substrate not detected. The nutrient content and pH of the adipose surface is similar to that of DFD meat and, providing the surface is moist, the spoilage pattern is similar to that of DFD (Nottingham *et al.*, 1981).

Meat as a product

Table 1.2 lists factors affecting meat quality. In terms of consumer acceptance the two most important aspects of meat rejection are the amount of meat exudate and the colour.

WATER HOLDING CAPACITY

Water in muscles is held between the actin and myosin microfilaments within the sarcolemma surrounding the myofibrils. On conversion from muscle to meat, shrinkage occurs as ATP is exhausted and myofilaments are cross-linked. The ultimate pH (5.4-5.5) of normal meat is near the isoelectric point of many of the muscle proteins. A reduction in

both the negative charge and electrostatic repulsion caused by the fall in pH during *rigor* decreases the water holding capacity *vis-à-vis* to that *in vivo* (Hood and Mead, 1993). This together with shrinkage results in an increased exudate or drip. There is a notable absence of drip from electrically stimulated meat; the reasons for this are unknown (Hood and Mead, 1993).

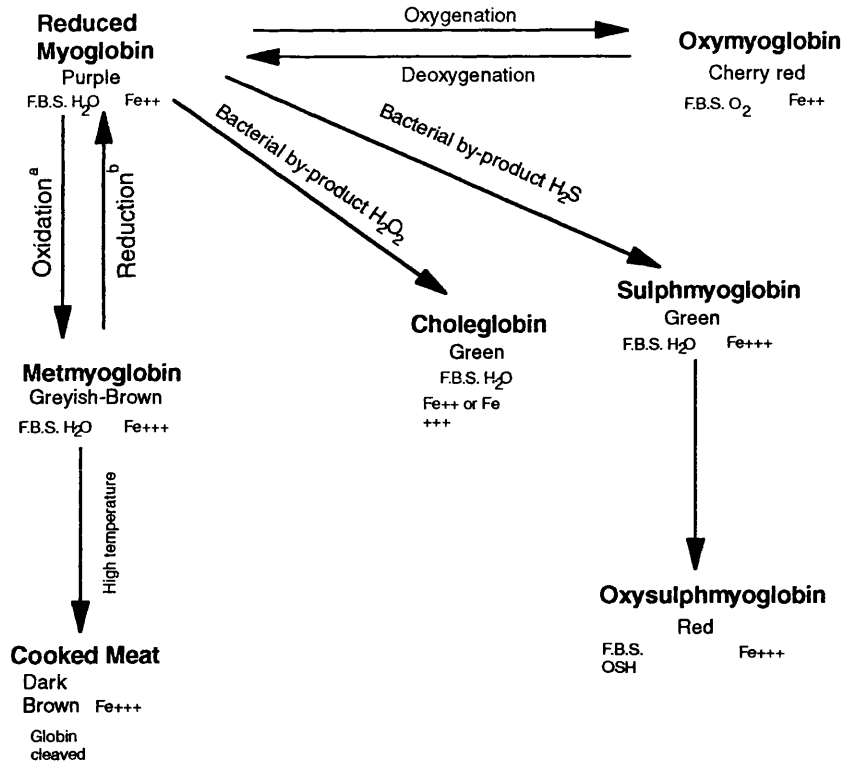
Table 1.2 Factors affecting the quality of meat

Major Influence	Additional Factors
Nature of the carcass meat	pH Aw Additives/preservatives Respiration rate (dependent on species, health, handling, slaughtering, chilling, sanitation, character of the muscle tissue, packaging type, distribution and storage)
Temperature	Slaughter Ageing procedure Storage
Colour	Relative proportions of myoglobin, metmyoglobin, oxymyoglobin, carboxymyoglobin, sulphmyoglobin and choleglobin
Micro-organisms	Load Types

MEAT COLOUR

Meat colour is of prime importance to consumers who relate it directly with freshness and quality (Seideman *et al.*, 1984, Taylor, 1985). Fresh beef is expected to be a bright cherry-red colour, whilst pork a duller purplish-red and lamb a darker red. These differences are due to the concentration of myoglobin (Seideman *et al.*, 1984). Pork, for example, has approximately one tenth of the myoglobin content of beef.

As most of the haemoglobin in the blood is removed during bleeding, the colour of meat is determined by the state of myoglobin (Mb), the primary pigment in muscle tissue. Myoglobin is a globular monomeric protein which protects a heme prosthetic group (Seideman *et al.*, 1984). The valence state of the iron atom and the nature of ligand/molecule attached to the free binding site of the pigment are responsible for the colour changes (Figure 1.3).



F.B.S. Free binding site

a Autoxidation - denaturation of globin gives no haem protection. Reaction increases with temperature, low pH and low pO_2.

b Metmyoglobin reducing activity caused by enzymes in the muscle

Figure 1.3 Forms of the myoglobin pigment present in meat
Information from Seideman *et al.* (1984)

There is a dynamic equilibrium between the different forms of myoglobin in muscle tissue; it is affected by many factors (Table 1.3). Generally meat has three pigment layers, an inner purple layer (myoglobin), a thin brown intermediate layer (metmyoglobin) and a red outer layer (oxymyoglobin) (Seideman *et al.*, 1984). Typically the metmyoglobin content is most pronounced about 2 mm below the surface (Hermansen, 1984). Metmyoglobin formation must be prevented in order to extend the shelf-life of meat. There may be colour variation over the surface of the meat. In some cases this is due to bacterial growth, patches of biofilm preventing oxygen diffusion to the meat surface (Seideman *et al.*, 1984). With high pH meat the muscle fibres are swollen and tightly packed thereby impeding absorption of light. The high pH does not allow oxygen to combine with myoglobin. A very thin layer of oxymyoglobin results. These two factors combine to give the meat a dark appearance, hence its name, dark, firm, dry (DFD) meat (Seideman *et al.*, 1984). Consumers associate the brown colour of metmyoglobin with 'old' meat. A level of 20% metmyoglobin at the surface of meat causes sales to drop by a factor of 2 (Renerre, 1990).

Table 1.3 Factors affecting the equilibrium between the forms of myoglobin

Intrinsic	Extrinsic	Technological
Animal species	Treatment of animal pre-slaughter	Packaging[c]
breed	during slaughter	Distribution
age	Processing e.g. hot-boning	Storage
sex	electrical stimulation	temperature[d]
diet	chilling rate	light[e]
Muscle type[a]		micro-organisms
Rate of *rigor* onset		
pH of muscle[b]		

a high-use muscles have high myoglobin levels
b high pH increases respiration rate and therefore creates an atmosphere low in oxygen
c metmyoglobin formation is prevented with no or high O_2
d the rate of discoloration doubles every 5 °C
e UV light causes Mb oxidation
Information from Renerre, (1990)

It has been reported that *Chromobacterium violaceum, Kurthia zopfii* and a strain of *Lactobacillus fermentum* affect meat colour. Metmyoglobin was converted to oxymyoglobin (*K. zopfii*) or to nitric oxide myoglobin (*Ch. violaceum and Lact. fermentum*), a bright pink pigment common in cured meats (Arihara *et al.*, 1993). The latter change is particularly noteworthy as lactic acid bacteria are generally regarded as "safe" and are frequently used in starter cultures for meat products. There is potential therefore for their inoculation to retain the colour of meat surfaces.

Spoilage of fresh red meats

MICROBIAL CONTAMINATION

The deep muscle tissue is generally considered to be sterile (Gill, 1979), although the captive bolt used to stun animals or the pithing rod may introduce occasional contaminants which are transported by the bloodstream to the spleen and sometimes the muscles (Grau, 1988). There is some evidence also that micro-organisms may penetrate the muscle between the fibres and endomysium of muscle due to the separation of their surfaces during *rigor* (Gill *et al.*, 1983).

Micro-organisms on carcasses come from a variety of sources - the environment, animal feeds and the animal itself (see Figure 1.1). These are transferred to the meat surface during processing either via the hide or faecal contamination as well as from the structural surfaces, processing equipment and the general environment, particularly the floor of the abattoir. The hands and aprons of workers also play an important role in contamination. Newton *et al.* (1978) found over a one-year period that counts on carcasses were regularly 0.3% of those on the hides. Thus contamination from this depot is very important. Structural surfaces in meat-dressing areas were also found to have large bacterial loads.

Gram negative organisms formed a high proportion of the airborne flora in the immediate vicinity of the mechanical hide pullers in one slaughterhouse (Gustavsson and Borch, 1993). A microbial flora of similar composition was noted on meat excised from the carcasses following storage.

In the main mesophilic organisms are transferred initially to meat (Dainty, 1989). The incidence of psychrotrophic bacteria in the environment, and therefore on the hides, varies with the ambient temperature and probably with the level of rainfall. Psychrotrophs are more common than mesophiles in wet, cold conditions (Newton *et al.*, 1978). The organisms most commonly isolated from dressed meats are *Acinetobacter, Arthrobacter, Aeromonas, Alcaligenes, Bacillus, Brochothrix, Clostridium*, coryneforms, various genera of Enterobacteriaceae, *Flavobacterium*, lactic acid bacteria, micrococci, *Moraxella, Pseudomonas, Psychrobacter, Staphylococcus, Streptococcus, Vibrio* and yeasts/moulds (Gill and Newton, 1978; Ahmad and Marchello, 1989; Dainty, 1989; Vanderzant and Nickelson, 1969). Pseudomonads, the major spoilage organisms of chilled meat exposed to O_2, were found to be common members of the microflora in meat processing environments (Nortjé *et al.*, 1990 a,b; Gustavsson and Borch, 1993; Gill and Bryant, 1992). In beef abattoir/processing plants, a resident flora of *Pseudomonas fluorescens* was found in the chillers, the highest incidence being in the condensate on pipes beneath the ceiling and on the surfaces of doors. The depots of infection obviously determined the incidence of contamination on meat such that after chilling *Ps. fluorescens* > *Ps. lundensis* > *Ps. fragi* > strains of *Acinetobacter*. *Vibrio* spp., which made up 19% of the airborne flora, were not recovered from meat (Gustavsson and Borch, 1993). Gill and Bryant (1992) found a relatively high incidence of the *Pseudomonas/Acinetobacter/Moraxella* group in only one of three pig processing plants, micrococci being dominant in the other two. The differences in the observations of these workers and those of Gustavsson and Borch (1993) may be due to the different species of animal harbouring a particular resident flora. Fresh meat has a bacterial load of 10^2 - 10^3 g^{-1} if hygiene is optimal; 10^3 - 10^4 g^{-1} is general after dressing in British abattoirs (Anon., 1991).

Decontaminating sprays

After evisceration carcasses are commonly washed to remove blood, bone-dust, hair and soil (Grau, 1988). Ellerbroek *et al.* (1993) studied the effect of a water spray on lamb carcasses. The numbers of bacteria on the highly contaminated ventral area did not decrease whilst the dorsal area, normally relatively free of bacteria, harboured a significant

microflora. Antimicrobial organic acids (Baird-Parker, 1980, Brown and Booth, 1991) have been suggested for use in decontaminating rinses both after skinning and after evisceration and splitting (Anon., 1993). Sprays containing acid have been shown to reduce contamination of beef (de Zuniga *et al.*, 1991), lamb (Egan *et al.* 1991), veal (Smulders and Woolthius, 1985) and buffalo (Surve *et al.*, 1991). The water pressure, flow rate (de Zuniga *et al.*, 1991), concentration (Surve *et al.*, 1991) and type of acid (Young and Foegeding, 1993) determine the extent of decontamination. Ziauddin *et al.* (1993) observed a decrease in the numbers of *Staphylococcus aureus, Salmonella newport, Bacillus cereus, Streptococcus* (now *Enterococcus*) *faecalis* and *Pseudomonas fragi* following the addition of 1-2% lactic acid (final concentration) to spray water. As these organisms were incubated at near-optimal growth temperatures, the effect of storage at chill temperatures on their viability was not determined. Solutions of alkali have been suggested also for use as sprays for the decontamination of animal carcasses (Rhone-Poulenc Patent, 1992).

BACTERIAL SPOILAGE OF CHILLED FRESH MEATS STORED IN AIR

As noted previously, in a commercial context consideration need only be given to surface contaminants. The moist, nutritious surface (Table 1.1) of meat is conducive to the growth of a range of bacteria. *Clostridium* spp. spoil meat kept at >25 °C. A consortium of bacteria, commonly dominated by *Pseudomonas* spp., are usually responsible for spoilage at -1 - 25 °C providing the atmosphere is "moist". Odour and slime production causes spoilage in 10 d at 0 and 5 d at 5 °C (Hood and Mead, 1993). The organisms, substrates used, and odours formed are listed in Table 1.4. With a dry meat surface fungi (often *Thamnidium, Cladosporium, Sporotrichum*) are selected (Ingram and Dainty, 1971, Gill and Newton, 1980b). In the UK it would be very unusual to find meat stored at more than 25 °C, or under conditions conducive to fungal growth.

With storage, the odour of meat changes gradually from a fresh "meaty" smell ($\leq 10^7$ bacteria g^{-1}) to an inoffensive but definitely non-fresh one, to a dairy/buttery/fatty/ cheesy (10^8), and eventually to a sweet/fruity and finally to putrid ($>10^9$) odour (Dainty *et al.*, 1985). Slime becomes evident when the bacterial load is about 10^8 g^{-1}; immediately before this the meat surface has a tacky feel (Ingram and Dainty, 1971). A deterioration of the colour of the meat due to a fall in the partial pressure of oxygen (Lambert *et al.*, 1991; Nychas *et al.*, 1988) under patches of micro-organisms (Gill and Molin, 1991) may cause customers to reject meat before spoilage is fully manifested.

Table 1.4 Substrates used and volatiles produced by micro-organisms growing on meat stored aerobically at chill temperatures.

ORGANISM	SUBSTRATE*	VOLATILE	ODOUR
Pseudomonas (usually dominant)	Glucose[1] Amino acids[2] Lactic acid[2]	Ethyl esters Methyl ethyl esters S-compounds C_{11} aliphatic hydrocarbons Ammonia Putrescine	fruity putrid
Brochothrix (sometimes dominant)	Glucose[1] Amino acid (glutamate)[2] Glycerol[2a] Glycerol-3-phosphate[3b] Inosine[4b] Ribose[a]	Acetoin/Diacetyl Branched chain alcohols Acetic acid 3-methyl butanol 3-methyl butanal Butanediol	Sickly Sweet Dairy/ Butter
Enterobacteriaceae (sometimes important)	Glucose[1] Glucose-6-phosphate[2] Amino acids[3] Lactic acid[3]	Branched chain esters S-compounds Amines H_2S	Putrid
Acinetobacter *Moraxella* (minor contaminant)	Amino acids[1] Lactic acid[2]	Esters Nitriles Oximes Sulphides	None reported
Psychrobacter immobilis (rarely found)	No details	No details	No details
Shewanella (minor proportion)	Glucose[a] Amino acids[a,b] (serine & cysteine) Lactic acid[c]	Sulphides	Putrid/ faecal
Lactic Acid Bacteria (*Lactobacillus*) (minor proportion)	Glucose[1] Amino acids especially arginine[2]	Fatty acids H_2S Methanethiol Dimethyl sulphide Ethanol	Sour

* Order of utilisation 1 - 4 normal pH meat and a - b high pH meat

Based on information from: Ingram and Dainty (1971), Gill (1976), Gill and Newton (1977), Gill and Newton (1978), Gill and Newton (1979), Dainty and Hibbard (1980), Dainty *et al.* (1985), Gill (1986), Edwards *et al.* (1987a), Grau (1988), Dainty (1989) and Dainty and Mackey (1992)

Initially glucose [the key determinant of the rate of spoilage (Table 1.5)] is utilised for growth, but as the bacterial population approaches the carrying capacity (>10^8 cfu g^{-1}) the diffusion gradient from the underlying tissue to the surface of carcass meats fails to match the microbial demand. Other substrates are then used sequentially (Gill and Newton, 1977; Gill and Newton, 1978) until finally nitrogenous compounds lead to the formation of malodorous substances. This is of course a simplified overview. Antimicrobial compounds produced by some organisms (recently reviewed by Stiles and Hastings, 1991), the

importance of microclimates in different areas on the meat surface (Dainty *et al.*, 1979) as well as the metabolic attributes of particular organisms will contribute also to the rate and extent of spoilage.

Table 1.5 The effect of additional glucose concentration on the shelf-life of minced beef

	Glucose concentration (% w/w)		
	0	2	5
Minimum pH	5.6	5.2	5.1
Days to reach minimum pH	0	9	11
Days to reach 10^6 cfu g^{-1}*	5	14	20

* 10^6 cfu g^{-1} was taken as an indicator of the onset of spoilage
Data taken from Shelef (1977)

In high pH meat (DFD), the glucose level is low (0-33 µg g^{-1} wet weight of meat at pH 6 - Gill, 1982), and the amino acids are used more quickly by *Shewanella putrefaciens, Aeromonas* spp., *Serratia liquefaciens, Yersinia enterocolitica* and lactic acid bacteria. Hydrogen sulphide production may lead to spoilage odours and the production of sulphmyoglobin (with associated greening of the meat). Both events lead to more rapid spoilage (Gill and Newton, 1979; Newton and Gill, 1980-81; Hood and Mead, 1993). With aerobic storage at 6 °C, DFD beef emitted off-odours after two days whereas that of normal pH did so after four days (Bem *et al.*, 1976). As would be expected the addition of glucose to DFD extended the shelf-life (Gill and Newton, 1979).

Pseudomonas spp.

Pseudomonads tend to dominate the microbial consortium in aerobically stored meats (Harrison *et al.*, 1981). Such dominance may be due to a fast growth rate associated with their high affinity for oxygen (Newton and Gill, 1978). Farber and Idziak (1982) suggested that the success of this bacterial group may be due also to their mode of catabolism of glucose. With most strains of pseudomonads from meats glucose is converted extracellularly to gluconate or to 2-ketogluconate. These compounds,which are not commonly used by competing organisms, serve as extracellular energy reserves and are transported into the pseudomonad cell when glucose becomes limiting. In an oxygen-limited environment (*i.e.* when the cells are in high numbers), glucose metabolism by *Ps. aeruginosa* was shown to occur via the intracellular phosphorylative rather than the extracellular route (Mitchell and Dawes, 1982).

When the glucose concentration is limited or the partial pressure of oxygen is low , and lactate has been exhausted, pseudomonads attack amino acids (Gill, 1986). This results

in the production of malodorous volatiles. Dainty, Edwards and Hibbard (1984) studied the different species of *Pseudomonas* isolated from beef (Shaw and Latty, 1982). They found large differences between strains (Table 1.6).

Table 1.6 The odours and associated volatiles produced by representative strains of meat pseudomonads*

Organism	Odour	Volatiles produced
*Ps. fragi***	cheesy ambrosia creamy rancid	Dimethylsulphide Ethyloctanoate Compounds present in uninoculated meat but in higher concentrations in the pseudomonad inoculated meat
*Ps. fragi****	strong fruity	Ethyl esters of C_2-C_8 fatty acids, Methyl esters
Ps. fragi	garlic/oniony	Isopropylthiol (high levels) Derivatives of Dimethyldisulphide, Dimethyltrisulphide and thioacetate Ethyl esters of C_2-C_8 FA
Ps. lundensis		Ethyl esters of C_6-C_8 FA. Derivatives of methane
Cluster 4 (Unidentifed)		Dimethylsulphide Compounds present in meat but in higher concentrations in the pseudomonad inoculated meat

* Information from Dainty, Edwards and Hibbard (1984)
** Strains from the study of Shaw and Latty (1982) *** *Ps. fragi* formed three separate clusters

Proteolytic bacteria have the potential to penetrate tissue (Gill and Newton, 1978) but, amongst the meat spoilage bacteria, only *Pseudomonas* and *Aeromonas* spp. have demonstrable proteolytic activity (Dainty *et al.*, 1975). With pseudomonads, however, relatively little protease is produced at a pH less than 6.7 (Dainty *et al.*, 1975). It is produced initially at the mid-exponential and reaches a maximum in the early stationary phase of growth at a time when spoilage is already evident. The optimum temperature for protease production by *Ps. fluorescens* is 20 °C. Easily metabolisable compounds repress protease production in pseudomonads (Fairbairn and Law, 1986) with high concentrations of 'free' amino acids causing catabolite repression of such enzymes during the exponential phase of growth (Fairbairn and Law, 1987). It may be assumed that in most circumstances these factors combine to inhibit protease action. Even though hydrolytic enzymes may be of minor importance in spoilage *per se* proteases and lipases produced by *Pseudomonas fragi* have been shown to have a detrimental effect on beef colour (Bala *et al.*, 1979).

The dominant role of pseudomonads in meat spoilage may not be due entirely to growth rate alone. Some pseudomonads have been shown to inhibit other bacteria; *viz. Ps.*

fluorescens inhibited *Staphylococcus aureus, Escherichia coli, Salmonella typhimurium, Citrobacter freundii* and *Klebsiella* spp. *Salmonella typhimurium* was inhibited weakly by *Ps. fragi.* The inhibition was attributed to iron deprivation (Freedman *et al.*, 1989). A recent study involving 209 pseudomonad strains isolated from fish identified 67 strains which inhibited one or more of six test strains of bacteria. The addition of iron negated the inhibition of the majority of these. Over 80% of the inhibitory strains produced siderophores in comparison with approximately 10% of the non-inhibitory strains (Gram, 1993). Some strains did, however, cause inhibition when grown in a medium having high iron concentration. The mechanism was not elucidated, but the prospect of the pseudomonads producing a bacteriocin, antibiotic or other inhibitory agent must be considered.

Brochothrix thermosphacta

At 5 °C *Brochothrix* reached a maximum population of 10^9 after 9d on beef of normal pH (Campbell *et al.*, 1979). On high pH (>pH 6.0) meat there was a diphasic growth curve; the initial rapid growth of this species was associated with glycerol and ribose metabolism and the subsequent slower growth with glucose-3-phosphate and inosine utilisation. Hypoxanthine produced from inosine was shown to be inhibitory; it probably contributed also to the reduced growth rate (Grau, 1988). Dainty and Hofman (1983), who studied fifteen different isolates of *Br. thermosphacta,* noted variations in the proportions of the volatiles produced by individual isolates. Generally, butanediol was produced until glucose was depleted. Isovaleric and isobutyric acids and 3 methylbutanol were detected when 60% of the glucose had been metabolised.

Enterobacteriaceae

Although members of this family are common but minor contaminants of meat, little attempt has been made to identify their contribution, if any, to organoleptic changes. *Serratia liquefaciens,* together with *Enterobacter aerogenes* and *Citrobacter* spp., were found on lamb chops (Newton *et al.*, 1977b). The first named species was found to produce an ammoniacal odour (Dainty *et al.*, 1975).

Acinetobacter/Moraxella/Psychrobacter

Small numbers of members of this complex occur on stored meat but they fail to compete effectively with pseudomonads. Although *Acinetobacter* would compete with

pseudomonads for amino acids and lactic acid, their low oxygen affinity is such that the pseudomonads become dominant (Baumann, 1968). Contamination of meat with high numbers of *Acinetobacter/Moraxella* should be avoided as they may reduce the partial pressure of oxygen, allowing pseudomonads to utilise amino acids and cause spoilage odours (Gill, 1986).

Others

Shewanella putrefaciens (formerly *Alteromonas putrefaciens*) can be of high spoilage potential even if it is not the dominant species (Gill, 1986). It is more plentiful in fish and broilers than in meat - a possible link to the presence of water - a habitat in which these organisms are more numerous than on/in the abattoir environment. It is not found on normal pH meats but may be a problem in DFD meats. *Shewanella putrefaciens* may be able to grow using iron as an electron acceptor. Several constituents of a pathway for the reduction of manganese (Figure 1.4) are also present in meat, *viz.* acetate from microbial breakdown of glucose, $Fe_{(II)}$ - $Fe_{(III)}$ in myoglobin and sulphur containing amino acids are a source of H_2S. The organism is associated with odour production and a greening of meat (sulphmyoglobin formation).

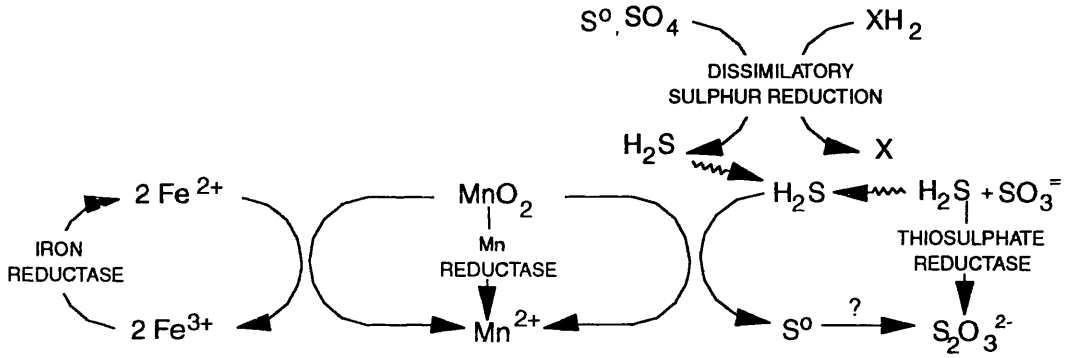


Figure 1.4 **Potential for growth of *Shewanella putrefaciens* on meat in anaerobic atmospheres using iron as electron acceptor**
Adapted from Nealson and Myers (1992)

MODIFIED ATMOSPHERE PACKAGING

Introduction

Modified atmosphere packaging extends the shelf life of meat, minimises spoilage losses, opens up new markets and provides a convenient packaging method for large scale distribution (Genigeorgis, 1985). It offers the supermarket a pack comparable to a grocery product allowing consumer selection with rigorous stock rotation. The method of packaging

involves the use of gas-impermeable film and the introduction of a gaseous mixture differing from that of air [78.08% nitrogen, 20.95% oxygen, 0.93% argon, 0.03% carbon dioxide and small quantities of ozone and inert gases (Collins English Dictionary)]. As stated previously vacuum packing is included in MAP; the trapped atmosphere is enriched with CO_2 derived from meat and microbial metabolism, the remaining atmosphere being nitrogen (Hood and Mead, 1993).

Methods

In MAP, meat is placed on a rigid pre-formed tray. A heat-sealable film is placed over the tray and the atmosphere modified by evacuation and/or flushing with the appropriate gas mixture before sealing. Close contact of meat and the web is an essential feature of vacuum packing if shelf life is to be extended. Four methods are used to achieve this end (Taylor, 1985; Anon., 1987a):

1) The meat is placed in a pouch and evacuated in a double-vacuum chamber then sealed with a metal clip. Heat-shrinkage at 90 °C ensures maximum contact of the meat and web.

2) Meat is placed in a bag which is then evacuated and heat sealed. On return to atmospheric pressure, the bag collapses round the meat. Colour deterioration may be a problem with meat exposed to pockets of residual gas. Unsightly accumulation of "drip" in the corners of the pack may also cause rejection by the consumer.

3) With automatic thermoforming machines, a web of plastic is used to line a tray, the meat added and an upper web sealed under vacuum. This method is good for bone-in joints and for meats which are subsequently cooked in the plastic film.

4) Vacuum skin packaging (VSP) - A web is heated, draped over the product in a vacuum chamber, and the films are sealed whilst still under vacuum (Anon., 1987a). In the USA, over 80% of beef, 35% of lamb and 5% pork is shipped in this manner (Williams, 1988). VSP can give a shelf-life of 14 weeks at 0 °C (8 weeks for high pH meat). The efficacy of VSP was compared to MAP for storage of pork and beef. Off-odours occurred more rapidly in MAP than in VSP but colour retention was better with the former (Taylor *et al.*, 1990).

An oxygen impermeable, or commonly, a freely permeable film is used in VSP. Marks and Spencers, for example, use oxygen-permeable VSP with an external pack of MAP in the "Fresh for home freezing" range. This ensures control of the shelf-life until the consumer freezes the product (Rice, 1990).

The CAPTECH system was developed to extend the shelf-life of beef and lamb to more than 16 weeks. This allows shipment of fresh meat for example from New Zealand to

the UK. The atmosphere in a tough metallised (e.g. aluminium) laminated barrier bag containing the meat (Gill, 1989) is modified with multiple gas flushing, using a snorkel system, which reduces the level of oxygen to <0.1% (Gill, 1989). Normal evacuation of a pack results in a residual 1% of oxygen. With the snorkel system less than 0.05% O_2 remains and a long shelf life is achieved (Gill, 1990). The CAPTECH process can extend the shelf life of beef and venison to 5 and chicken and pork to 3 months (Bentley, 1991) providing the temperature of storage is -1 °C. This advanced packaging system may accentuate drip and cause small cracks in the meat surface. Of all the potential spoilage organisms, *Brochothrix thermosphacta* is the one that may not be completely inhibited by the system.

Gas Exchange Preservation is a technology being developed in Australia. Air is pumped out of the pack and other gases are introduced in rapid succession for particular purpose e.g. CO for colour, SO_2 or ethylene oxide to kill the bacteria and finally N_2 to flush the pack. (Church, 1993). Safety legislation regarding toxic gases remains a problem.

Films

The monomer unit dictates the attributes of polymeric films used in food packaging. Simple carbon and hydrogen films (polythene) are effective barriers to water thereby preventing moisture loss (with overwrapped packs). The inclusion of chlorine in a film significantly reduces gas transmission but increases brittleness (Taylor, 1985). In the UK, the most common films are a base web of unplasticised polyvinyl chloride/polyethylene and a top web of polyvinylidene chloride-coated polyester/polythene with an antifog agent - required in films used in retail display to prevent condensation during temperature cycling in refrigeration units (Greengrass, 1993). Meat temperature in retail display can be higher than ambient due to the greenhouse effect caused by radiant energy from the lights (Hood and Mead, 1993).

Specific properties of a film can be modified further by the inclusion of particular monomers - e.g. acetals/acrylates to increase the plasticising properties (Hood and Mead, 1993) or by the incorporation of additives that scavenge or release carbon dioxide and oxygen or other preservatives (Bennett, 1993). A patent has been sought for a film causing aerobic conditions to be established when the pack undergoes temperature abuse (Patterson and Cameron, 1992).

The importance of gas transmission rates of plastic films was demonstrated by Newton and Rigg (1979). They found that shelf-life was inversely proportional to oxygen

permeability. Film permeability is expressed in theoretical gas transmission rates at specified temperature and humidity. Plastic-film laminates with low oxygen transmission rates (OTR) of <1ml m^{-2} 24 $h^{-1}atm^{-1}$ allow the permeation of small amounts of oxygen (Stiles, 1991). A metallised layer must be included in the laminate for a film to be an effective barrier to oxygen. This is commonly achieved with aluminium.

Difficulty in opening MAP and VP packs of meat is a common complaint from consumers. MAP are easier to open than VP. The situation may be ameliorated with the introduction of peelable films (Hood and Mead, 1993). These allow the package to be changed to MAP before the outer web is removed to give an aerobic pack. This method allows meat to "bloom" before retail display. The use of films has been questioned by Roberts (1992) in the context of the migration of potentially hazardous substances into foods. There are standard test methods for specific monomers and the overall migration limits which are tested with food simulants (e.g. distilled water, 3% acetic acid, 15% ethanol and rectified olive oil). Standards are specified by the EC (Tice, 1992).

Gases used and their effects

A combination of gases, commonly a mixture of oxygen, carbon dioxide and nitrogen, is normally used in modified atmosphere packaged meats,.

Oxygen This gas prevents the growth of anaerobic micro-organisms, thereby ensuring the safety of the product with regard to *Clostridium* spp. High levels of oxygen promote lipid oxidation and rancidity (Ochi, 1987). At intermediate concentrations it may stimulate the growth of aerobic bacteria but, if high concentrations (e.g. 80%) are used, a decrease in the growth rate of these organisms was noted. At pressures above 240mm there is an extension of the fresh appearance of meats. This is due to a decreased dissolution of O_2 from oxymyoglobin to myoglobin and a decreased reaction rate of myoglobin to metmyoglobin (Young *et al.*, 1988).

Nitrogen This inert, tasteless gas of low solubility is often used as a filler in MAP to reduce physical stress on the product and prevent pack collapse.

Carbon dioxide This water and lipid soluble gas inhibits product respiration. It is bacteriostatic also, extending the lag phase and/or increasing the generation time of susceptible bacteria. The effective concentration of CO_2 is dependent on the factors listed in

Table 1.7. With an aqueous system the overall reaction is $CO_2 + H_2O <=> H_2CO_3 <=> HCO_3^- + H^+$ (Dixon and Kell, 1989), the proportions being dependent on pH (Figure 1.5). Within the normal range of meat pH, CO_2 in solution is the predominant form. The solubility of CO_2 in meat increases by 360 ml kg^{-1} for each pH unit rise and approximately 19 ml kg^{-1} for each 1 °C rise between -1 and 10 °C (Gill, 1988).

Table 1.7 Factors affecting the concentration and activity of carbon dioxide in meat

Meat attributes	Intracellular effects	General	Bacterial
composition of meat	protein binding	pH	sensitivity
proportion of fat	metabolism	temperature	growth phase
size and shape of cuts		pCO_2	
composition of exposed surfaces		solubility of CO_2 in system	
		presence of other solutes	

Information from Gill, (1988); Jones, (1989); Stiles, (1991)

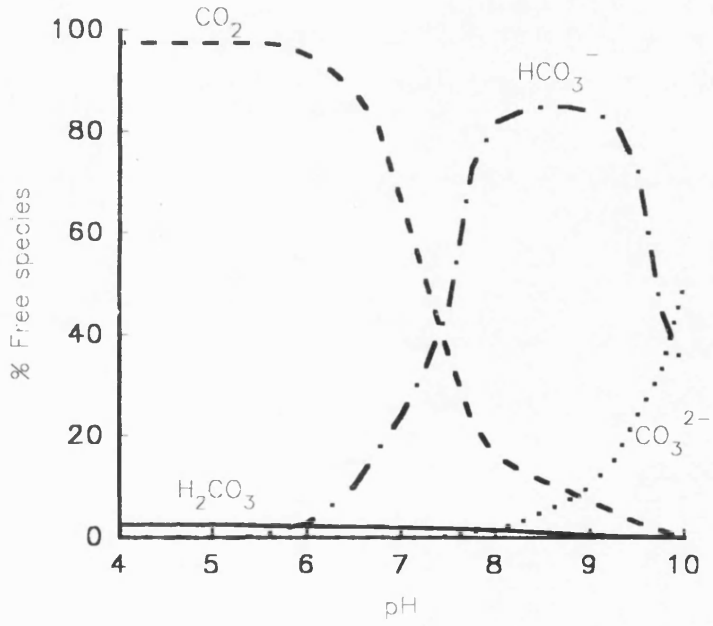


Figure 1.5 Proportions of dissolved carbon dioxide, carbonic acid, bicarbonate ions and carbonate ions as a function of pH. Data taken from Daniels *et al.* (1985)

The mechanism of bacteriostasis due to carbon dioxide is still unknown despite its use in food preservation for upwards of a century. The consensus opinion is of a synergistic action between some or all of the following in a product (Wolfe, 1980; Dixon and Kell, 1989; Jones, 1989; Farber, 1991; Lambert *et al.*, 1991):

1) amendment of membrane function (nutrient uptake)

2) inhibition of enzymes or a decrease in enzyme reaction rates

3) an intracellular pH change - perturbation of enzyme equilibria

4) alteration of proton gradient (*cf.* the effect of decreased internal pH)

5) a change in the physico-chemical properties of proteins due to a change in the internal electrostatic forces. The high reactivity of amines causes the formation of carbamic acids.

6) feedback inhibition - internal pH affecting decarboxylating enzymes thereby producing more CO_2.

7) dissipation of energy - completion of a futile cycle. An increase in carboxylation and decarboxylation reactions result in a net loss of energy.

As single strains have been used in studies attempting to determine the role of CO_2 in microbial inhibition, generalisations cannot be made. With facultatively anaerobic organisms grown in an environment enriched with carbon dioxide, the enzymes of respiratory metabolism were considered to be inhibited (Gill and Tan, 1980). Studies with *Ps. fluorescens* showed a number of effects of CO_2. The uptake of the majority of a number substrates was inhibited to various extents as was the activity of non-decarboxylating enzymes. The decarboxylating enzymes were less severely affected (Tan and Gill, 1982). These workers proposed a non-specific effect on substrate uptake, possibly related to an expansion of the hydrophobic regions of proteins in the cell membrane. They emphasised however the probability of interactions between many factors. In aerobic systems, 20-30% CO_2 causes maximum inhibition; in anaerobic situations, the inhibition increases progressively with increased CO_2 concentration (Gill, 1988).

Clark and Lentz (1972) found that carbon dioxide had to be present continuously in order to affect microbial growth. Silliker *et al.* (1977), who used this gas as a pre-treatment for pork, observed a residual effect; aerobic spoilage occurred at 4 days with controls whilst CO_2-treated meat spoiled after 7 days. The pressure of CO_2 applied to meat had a pronounced effect on shelf life - 41 days with 1 atmosphere of CO_2 was extended to >121 days with 5 at 4 °C. It did not effect greatly the composition of the microflora (Blickstad *et al.*, 1981).

High CO_2 alone can cause discoloration (browning at partial pressures greater than 0.2 atm), a drop in the pH and a concomitant increase in the amount of exudate, a "sharp" taste and collapse of the pack (Leeson, 1987). To ensure that the unsightly drip does not become obvious to the consumer, exudate in MAP packs containing high levels of CO_2 is absorbed in pads or trapped in the patterned bottom of the tray.

Carbon monoxide Carbon monoxide has been used experimentally with MAP meats. As it causes the formation of the bright red carboxymyoglobin, the fresh appearance of meat is retained for periods longer than that stored in oxygen. Clark *et al.* (1976) found that if 1% (v/v) carbon monoxide was present in packaged meat throughout storage, then the shelf life was extended as a consequence of a protracted lag phase and decreased growth rate of susceptible micro-organisms. The safety features of CO have not been fully tested as yet, and potential problems of using this gas during preparation of packs needs to be evaluated.

Ozone Ozone has been recommended for inhibition of mould growth but in practice is considered to inhibit Gram positive rods > cocci > Gram negative rods (Anon., 1980). It is relatively ineffective against bacteria in complex media (Genigeorgis, 1985). The main concern with use of this gas is the possibility of accelerated rancidity (Jones, 1989). It is involved in the destruction of amino compounds, the coagulation of proteins and inactivation of enzymes (Ingram and Barnes, 1954). The effect of ozone on the spoilage of beef was studied by Kaess and Weidemann (1968). Discoloration of the meat was observed with the application of > 0.6 mg m^{-3} of ozone and storage at 0.3 °C. At this level there was a small extension in the lag phase of their slow-growing strains of *Pseudomonas* spp.; the rapidly-growing ones were unaffected. It delayed the "slime point" - visible manifestation of microbial growth - from day 12-13 in air to between days 20 -24 with ozone.

The headspace in MAP

Gill and Penney (1988) determined the most efficient gas-to-meat ratio. This ratio was about 2l of gas per kg of meat. The level of residual oxygen in the headspace of "anoxic" packs is important. When various oxygen concentrations were included in packs having different headspace volumes, it was found that an initial level of >0.15% oxygen compromised the colour of beef and lamb but not pork (acceptable at 1%). Increasing headspace volumes tended to negate this problem but at a cost in terms of pack size - large head space = larger pack size (Penney and Bell, 1993). This problem may be overcome with oxygen scavengers such that the full potential of MAP is realised. "Ageless" - a sachet containing loose, finely divided iron powder - reduces O_2 to less than 100 ppm by the formation of non-toxic iron oxide. A similar methodology has been used to isolate nitrogen-fixing anaerobes, with activated iron wool being used to remove oxygen in sealed containers (Parker, 1955). Other proprietary brands of scavengers absorb oxygen with the concurrent production of equal volumes of CO_2 thereby preventing pack collapse (Smith *et*

al., 1990b). At present there is some consumer objection to the inclusion of sachets in fresh foods, probably due to suspicion of an intrusive addition to a pack.

MAP products

Many interacting factors determine the quality of any MAP product (Tables 1.2, 1.8 and 1.9). The use of high pCO_2 and very low pO_2 (best for microbial inhibition) leads to an unacceptable colour which may be avoided if meat is re-packaged for retail, a costly exercise. Use of high pO_2 to give a colour acceptable to consumers may result in a short shelf-life due to increased growth of micro-organisms.

Table 1.8 Factors affecting the quality of MAP and VP fresh meats*

Major Influence	Additional Factors
Gaseous environment	Level of CO_2
	Level of O_2
	Presence of CO or ozone
Package attributes	Headspace
	Permeability of film - and faults in the film
	Form
	Integrity - reliability of the packaging process and seal strength
	Dissolution of a gas

* Alli (1993), Genigeorgis (1985) & Gill (1990)

One of the major problems with MAP and VP is the detection of leaking packs. Non-invasive techniques for monitoring packs are needed (Dr R. Hart, pers. comm.). Distribution of MAP packs must be controlled carefully not only in terms of temperature but also in handling practices. For example, if meat in retail MAP packs touches the oxygen impermeable film, then an area of low oxygen tension and discoloration of the product results. Such factors as these have resulted in a marked growth in logistical distribution systems offered by specialist haulage firms to supermarkets.

Potential future developments in MAP/VP

Many adaptations and developments of MAP technology are under study (Table 1.10). Some of these attempt to combine different preservation methods - the hurdle effect (Leistner, 1985; Earnshaw, 1990, Ooraikul, 1993, Gould, 1992). As yet none is exploring the use of additives or colour enhancers. With current legislation, approval of such amendments would be restricted to meat products (Gill, 1989).

Table 1.9 Factors for and against the use of MAP and VP of fresh meats*

Packing method	Advantages	Disadvantages
MAP	Inhibition of bacterial and mould growth	Cost
	Ease of opening (*vis à vis* VP)	Large headspace required
	Appearance of the cuts	Condensation - antifog agents required
	No compression	Pack versatility - sizes and shapes
	Bone-in-cuts easy to handle	"Rich"[a] packaging
		Careful handling required
		Drip loss - use of absorbent pads and/or patterned trays
		Packing line speeds low
		Difficulty of opening (*vis à vis* overwrap)
		Unsuitable for freezing (rancidity)
VP	Able to withstand abuse	Unsuitable for certain cuts and bone guards required
	Low cost	Colour deterioration patchy
	Rapid throughput	Pockets of drip
	Greater control over pack appearance	Close contact of meat and web required
		Colour unacceptable to consumer
		Slices stick together
		Undesirable "squashed" appearance
MAP & VP	Increased shelf life	Temperature control
	Retention of moisture - reduced shrink	Possibility of rejection of good meat because of colour
	New markets - better distribution	Detection of leakers a problem
	Decreased economic losses	

a A large headspace gives the impression to some consumers of an expensive packaging *vis-à-vis* the cost of the product
* Information from : Alli (1993), Chomon (1987), Taylor (1985), Muller (1986), Egan *et al.* (1991)

Beef in MAP packs has a shelf-life of more than 2 months at 1 °C and lamb and pork up to 6 weeks (Taylor, 1985). Shelf-life is determined by the choice of atmosphere, storage temperature and the meat type (Table 1.11 - for further information see Table A1.1 in Appendix 1). Spoilage of vacuum packed meat occurs between 10 - 12 weeks at 0 °C (Dainty, 1989) providing the meat is of normal pH and produced and processed under hygienic conditions, temperature control is adequate and a low oxygen permeability film is used (Egan, 1984). With storage a sour/acid/cheesy odour develops, due to the production of organic acids from carbohydrates by lactic acid bacteria (Egan *et al.*, 1991). Volatile compounds produced in MAP/VP are listed in Table 1.12. With VP, spoilage occurs after bacteria have attained maximum numbers (Madden and Bolton, 1991). Greening and a sulphurous odour due to H_2S production by some species of LAB (Dainty, 1989) may be evident also. Reactions within the meat may also occur and cause a bitter/liver-like odour and taint of the meat (Egan *et al.*, 1991).

Table 1.10 Future developments of MAP/VP

Development	Hurdle	Notes	Ref(s)
Natural preservatives	✓	As an additive or by inoculation/ selection of appropriate organisms	1
Organic acids	✓	As a decontaminating spray*/dip or by selection of LAB**	2,3,4
Bacteriocins	✓	Added in a pure form or by inoculation/selection of LAB**	5,6
Irradiation	✓	Decreases microbial load and meat reactions	7,8
and acid addition	✓	Adds another selective pressure	9
High pressure	✓	For reduction of microbial load - colour rejection causes problems	10
Bacteriophage	✓	Reduction of particular bacteria e.g. pseudomonads	11
Glucose addition		To extend the shelf-life of aerobic or MAP DFD meats	12,13
Anti-oxidants		For further colour stability e.g.sodium erythorbate, ascorbic acid	14,15
Edible films with or without additives		Reduction of exudate	16, 1
Time-temperature Indicators		Records temperature history	17

* see section on microbial contamination above (pp. 9) **Localised effects in meat from production by LAB
Sources: 1, Ooraikul (1993); 2, Baird-Parker (1980); 3, Brown and Booth (1991); 4, Labadie *et al.* (1975); 5, Stiles and Hastings (1991) 6, Kim (1993) 7, Lambert *et al.* (1992); 8, Grant and Patterson (1991b); 9, Farkas and Andrássy (1993); 10, Carlez *et al.* (1993); 11, Greer (1986); 12, Shelef (1977); 13, Newton and Gill (1980-81); 14, Gill and Molin (1991); 15, Manu-Tawiah *et al.* (1991); 16, Farouk *et al.* (1990); 17, Taoukis *et al.* (1991)

Bacterial spoilage of modified atmosphere packaged meats

As the numbers of bacteria (particularly pseudomonads) are restricted by the relatively high concentration of CO_2 the spoilage of VP meat occurs later than that stored aerobically. Homo- and heterofermentative LAB (for example *Lactobacillus, Leuconostoc, Lactococcus* and *Carnobacterium* spp.) typically develop on meat under enriched CO_2 atmospheres. Huffman *et al.* (1975) did not recover lactic acid bacteria from meat stored in 100% CO_2. The lactic acid produced by these bacteria inhibits Enterobacteriaceae, *Br. thermosphacta* and *Shew. putrefaciens* (Schillinger and Lücke, 1987b). With high pH meat (>pH 6.0) Enterobacteriaceae and other facultatively anaerobic bacteria (e.g. *Shewanella, Brochothrix*) may form an important part of the flora (Gill and Penney, 1986). Erichsen and Molin (1981) found that there was a mixed population including *Pseudomonas* spp., lactic acid bacteria and *Brochothrix* in MAP meat, whilst *Brochothrix* comprised 40% of the flora in VP and homofermentative lactobacilli were the predominant bacterial group in CO_2 packaged meat even in that having an initially high pH. As adipose tissue absorbed meat juices during processing (pp. 6), it manifests the spoilage characteristic of DFD meat when stored in MAP. With fat stored under VP, Patterson and Gibbs (1977) found a dominance of pseudomonads (55%) with 23% *Alcaligenes*, 9% LAB and 9% Enterobacteriaceae. On DFD

meat, Enterobacteriaceae constituted 41%, pseudomonads 36%, *Aeromonas* 9% and *Alcaligenes* 14% of the microflora.

In an anaerobic (or microaerobic) environment microbial competition centres mainly on glucose, glucose-6-phosphate and ribose (Gill and Newton, 1978). The dominance of a particular organism is determined by its relative affinity for a particular substrate (Newton and Gill, 1978). Growth, but not survival of pseudomonads is limited in very low oxygen conditions. If the impermeable film is removed, then the pseudomonads grow rapidly (Roth and Clark, 1972). Madden and Bolton (1991) found that in vacuum-packed beef, pseudomonads colonised the inner surface of the vacuum pouch, possibly due to the transferred oxygen being trapped at the meat-film interface. Growth was supported by carbohydrates in the meat exudate as well as catabolites of LAB metabolism.

Clostridium esterheticum (Collins *et al.*, 1992) has been isolated from spoiled vacuum packed beef on three occasions (Dainty, 1989, Kalchayanand *et al.*, 1989; Dainty and Mackey, 1992).

Pseudomonas

As pseudomonads require oxygen, their growth in anaerobic packs is limited. There have been some reports of their growth on VP meat (e.g. Egan, 1984). The growth of this group is reduced by the addition of carbon dioxide (Enfors and Molin, 1981), in 0.5 atm. carbon dioxide the growth rate of *Ps. fragi* was 50% of that in air. In microculture studies at 2 and 6 °C no growth was detected in CO_2, but small amounts were noted in VP and N_2 (Eklund and Jarmund, 1983).

Table 1.11 Examples of the spoilage of MAP red meats

Meat		Storage characteristics				Storage characteristics			Ref.
Species	Cut	Temp. °C	Atmosphere	OTR[a] of film	Gas to Meat ratio	Shelf life[b] (days)	Cause of spoilage[c]	Dominant organisms[d]	
Beef	Roasts	1-3	VP*	32		34	O	Lb^{si}	1,2
			100% O_2		1:1	13	O/C	Ps/Lb	
			20%CO_2 + 80% N_2			34	O/C	Lb	
			50% CO_2 + 50% O_2			27	O/C	Lb	
			20% CO_2 + 80% O_2			20	O/C	Lb	
			25% CO_2 + 25% O_2 + 50% N_2			20	O/C	Lb/Ps	
			51% CO_2 + 30% O_2 + 18% N_2 + 1% CO			34	O	Lb	
Lamb	Chops	-1	Air	N.S.	N.S.	14	CO	B/Ps^{si}	3
			80% Air + 20% CO_2			21	CF	B/Ps	
			80% O_2 + 20% N_2			21	CF	B/Ps	
			80% O_2 + 20% CO_2			21	CF	B	
			80% N_2 + 20% CO_2 Low O_2			42	C	B/Ps/Ent	
			80% N_2 + 20% CO_2 Oxygen free			56	F	B/Lb/Ent	
Pork	Roasts	1-3	VP	32		28	O	Lb^{si}	4,5
			100% O_2		1:1	14	O	Ps	
			20%CO_2 + 80% N_2			21	O	Leu	
			50% CO_2 + 50% O_2			14	O	Leu	
			20% CO_2 + 80% O_2			14	O	Leu/Ps	
			25% CO_2 + 25% O_2 + 50% N_2			14-21	O	Leu	
			51% CO_2 + 30% O_2 + 18% N_2 + 1% CO			14	C	Leu	

a OTR = Oxygen transmission rate. Measured in ml m^{-2} 24 h^{-1} at 1 atmosphere The temperature and relative humidity varies with film data.

b Where detailed the time is taken from the time of slaughter. The methods and times of ageing differ.

c O Off-odour, C Discolouration, F Off-flavour

d B *Brochothrix thermosphacta*, Ps. *Pseudomonas* spp., Leu *Leuconostoc* spp., Ent Enterobacteriaceae, Lb *Lactobacillus* spp., ^{si} selective media were used but isolates were taken from total counts were identified.

* VP Vacuum pack

Information from: 1, Christopher *et al.* (1979a); 2, Seideman *et al.* (1979a); 3, Newton *et al.* (1977a); 4, Seideman *et al.* (1979b); 5, Christopher *et al.* (1979b)

Table 1.12 The substrates used and volatiles produced by micro-organisms on meat

ORGANISM	SUBSTRATE*	VOLATILES	ODOUR
Lactic Acid Bacteria	Glucose[1] Amino acids especially arginine[2]	H_2S Methanethiol Dimethyl sulphide Ethanol	Sour
Brochothrix	Glucose[1] Ribose[a]	Acetoin/Diacetyl Branched chain alcohols Acetic acid	malty dairy caramel
Enterobacteriaceae	Glucose[1a] Glucose-6-phosphate[2b] Amino acids[3**] Lactic acid[3] inosine-mono-phosphate[w]	Sulphides Amines Diamines H_2S	Boiled egg Putrid/rotten
Shewanella	Glucose[1a] Amino acids[1,2a] (serine & cysteine)	Sulphides	Putrid/faecal

* superscripts show order of utilisation 1 - 3 use on low pH meat (normal) a - b use on high pH meat w weak growth

**except serine which may be used with glucose and glucose-6-phosphate, others used only after exhaustion of the other nutrients (Gill and Newton, 1979)

Based on information from:
Ingram and Dainty (1971), Gill (1976), Gill and Newton (1977), Gill and Newton (1978), Gill and Newton (1979), Dainty and Hibbard (1980), Dainty *et al.* (1985), Edwards *et al.* (1987b), Grau (1988), Dainty (1989) and Dainty and Mackey (1992), Newton and Gill (1978)

Lactic acid bacteria

Lactic acid bacteria in general do not produce malodourous substances (Dainty *et al.* 1975), but tyramine is produced by *Carnobacterium* (Edwards *et al.*, 1987b). Small concentrations of dimethylsulphide and methanethiol have been associated with the sour odour typical of vacuum packed/CO_2 stored meat stored for long periods (Dainty and Mackey, 1992). The growth and activity of a homofermentative *Lactobacillus* and a strain of leuconostoc on meat stored at 4 °C in 5% CO_2 and 95% N_2 were studied (Borch and Agerhem, 1992). A maximum population of 10^7 cfu g^{-1} was present after 2 weeks, at which time flavour changes were noted with the *Lactobacillus* sp. which produced acetic acid and D-lactate from L-lactate and glucose. Formate or ethanol was not detected, although some H_2S was evident towards the end of storage. An analogous flavour change was detected before the *Leuconostoc* sp. had attained maximum numbers. This organism produced D-lactate and ethanol from glucose. Some hydrogen sulphide was also formed but no malodour was detected in either case (Borch and Agerhem, 1992). A homofermentative rod attained a maximum population of 10^8 on VP beef. Off-flavours developed 7 to 13 days after this population had been achieved (Egan and Shay, 1982). Gill and Newton (1979)

found the generation time of a *Lactobacillus* sp. was unaffected by high or low pH meat in VP.

McMullen and Stiles (1993) identified the LAB species prevalent in MAP pork. *Lactobacillus alimentarius, L. farciminis* and *L. sake* were the three most common isolates. In VP or MAP (75% O_2 + 25% CO_2) beef stored for 14 or 28 days, leuconostocs were the dominant organisms (Hanna *et al.*, 1981).

Brochothrix thermosphacta

Variable growth of *Brochothrix* occurs under conditions simulating MAP or VP. Its growth is affected by the temperature, pH and gaseous atmosphere obtaining in the storage conditions (Gardner, 1981). In an anaerobic atmosphere, numbers decreased on low pH meat (Campbell *et al.*, 1979). Transfer after 2 days from aerobic to anaerobic conditions resulted in growth stasis (Campbell *et al.*, 1979). On high pH meat, however, 10^8 cfu g^{-1} were present after 10 days under anaerobic conditions at 5 °C (Campbell *et al.*, 1979) . The maximum yield depended on the substrate (Newton and Gill, 1978). The lag phase of *Br. thermosphacta* was longer than that of a *Lactobacillus* sp. or *Enterobacter* sp. under N_2 (Newton and Gill, 1978).

Enterobacteriaceae

Serratia liquefaciens and *Hafnia alvei* grew and produced cadaverine and putrescine in vacuum packed meat (Edwards *et al.*, 1985; Dainty *et al.*, 1986); *Pantoea* (formerly *Enterobacter*) *agglomerans* and *Klebsiella* grew, but did not produce the diamines. *Citrobacter freundii, Proteus* and *Yersinia* did not grow on similar meat in vacuum packs at 1 °C (Dainty *et al.*, 1986). Synergism was found between *Serratia* and *Hafnia* and those lactic acid bacteria utilising arginine to produce ornithine. Ornithine decarboxylase of the first two was involved in the conversion of ornithine to putrescine (Edwards *et al.*, 1985). Although arginine could be utilised, the Enterobacteriaceae generally do not contain the required dihydrolase (Holmes and Costas, 1992). In VP, Enterobacteriaceae produced branched chain esters, methanethiol and derivatives, dimethyldisulphide, dimethyltrisulphide, methylthioacetate and bis(methylthio)methane, the last being produced from high pH meat only (Dainty and Mackey, 1992). *Hafnia* also produced H_2S in mince (Hanna *et al.*, 1983).

Others

Shewanella putrefaciens grown on high pH VP meat produced dimethyldisulphide, bis(methylthio)methane and methylthioproponoate. A putrid/faecal odour was produced (Dainty and Mackey, 1992). This organism may cause greening (sulphmyoglobin) on VP DFD meat through H_2S production from cysteine. Its growth may be inhibited by addition of an acid to high pH meat - it is unable to grow on low pH meat (Gill and Newton, 1979). *Aeromonas* also is able to grow in high pH VP meat and produce putrid odours, probably due to methylthio-proponoate production (Dainty *et al.*, 1989). In VP meat *Yersinia enterocolitica* was recovered after 4 weeks and *Shew. putrefaciens* after 2 weeks storage at 0-2 °C. The latter reached 10^6 cfu g^{-1} by week 10 at which time greening was evident (Seelye and Yearbury, 1979).

Safety

There is no doubting that modified atmosphere packaging extends the shelf life and affects the spoilage microflora of meat. The safety of the procedure, however, is in doubt because of the potential growth of pathogens (e.g. Silliker and Wolfe, 1980; Genigeorgis, 1985; Hintlian and Hotchkiss, 1986; Farber, 1991), especially with the decreased growth of the competitive spoilage flora. The pathogens of concern in meats are: *Aeromonas* spp., *Clostridium* spp., *Escherichia coli, Listeria monocytogenes, Salmonella typhimurium, Staphylococcus aureus* and *Yersinia enterocolitica.* Hotchkiss (1988) proposed a "safety index" where the ratio of pathogen to spoilage organism growth indicated the relative safety of a packaging system. It is possible that the 'safest' atmospheres are ones which allow the growth of spoilage flora if the package is abused (Hintlian and Hotchkiss, 1986).

Packs having reduced oxygen tension may well be viewed with concern because of the potential for growth of *Clostridium* spp. There have been three reports to date of the isolation of *Clostridium esterheticum* (Collins *et al.*, 1992) from spoiled vacuum packed meats (Dainty, 1989; Kalchayanand *et al.*, 1989; Dainty and Mackey, 1992). Psychrotrophic strains of *Cl. botulinum* (types B and E) can be isolated from chilled environments having low oxygen tension (Gibbs *et al.*, 1994). The potential for growth of these organisms in chilled packaged meats must be established (Ooraikul, 1993).

The growth of pathogenic organisms will be affected by factors similar to those described previously for spoilage organisms. Temperature, pH and the ability of organisms to grow in CO_2 are important controlling factors in modified atmosphere packs (Table 1.13).

Retail foods have been surveyed for the presence of *Aeromonas* and *Yersinia* (Walker and Brooks, 1993). Overall, the incidence of isolation was 10.8 and 4.2% respectively. The proportions for fresh meats were, however, 57 and 32% respectively. The meat species were not described. These two genera have been found in relatively large numbers in pork packed in CO_2 (Blickstad and Molin, 1983 and Egan, 1984) and the growth of *Yersinia* at very low temperatures (Eklund and Jarmund, 1983; Gill and Reichel, 1989; Kleinlein and Untermann, 1990) is of obvious concern. In studies on *Yersinia* on beef steaks (Gill and Reichel, 1989) and mince (Kleinlein and Untermann, 1990) the numbers of spoilage organisms were always higher than those of the pathogen. In a study on *Yersinia*, *Aeromonas* and *Listeria*, packaging in CO_2 was more effective for inhibiting growth than vacuum packing (Gill and Reichel, 1990). At 5 °C in CO_2 *Yersinia* grew at 30% the rate of the spoilage flora and the other two species were inhibited. In vacuum packs, however, *Yersinia* grew at the same rate as the spoilage flora, *Aeromonas* grew at the same rate after an extended lag phase and *Listeria* at a decreased rate after an extended lag phase compared to spoilage organisms.

Table 1.13 Pathogens in MAP meat

Organism	Ubiquity	Disease	Effect of CO_2[a]	Minimum pH[b]	Minimum temperature (°C)
Aeromonas spp.	+++[c d e]	Gastro-enteritis	-[1 *]	5.5	-2[f]
Clostridium spp.	++	Various[2]	-/+	5.0	3.3[g 3 **]
Escherichia coli	+++	Diarrhoea	-	5.0	8[h]
Listeria monocytogenes	+++[i]	Various[4]	-/=	4.6	0[f]
Salmonella typhimurium	++	Gastro-enteritis	-	4.0	8[h]
Staphylococcus aureus	++	Gastro-enteritis	-	4.0[j]	10[k 2 *]
Yersinia enterocolitica	+++[d e]	Gastro-enteritis	-	4.6	-2[f]

a, Farber (1991); b, Jones (1989); c, Blickstad and Molin (1983); d, Gill and DeLacy (1991); e, Baker *et al.* (1986); f, Schofield (1992); g, Egan (1984); h, Genigeorgis (1989); I, Palumbo (1986); j, Gill and Reichel (1989); k Roberts (1982)
1, minimum temperature for toxin production; 2, *Cl. perfringens* - gastro enteritis, *Cl. botulinum* - death or very slow convalescence with symptoms of disturbance of vision, difficulty in speaking and swallowing, weakness and respiratory problems; 3, *Cl. botulinum* type E, other types have minimum temperatures of >10 and *Cl. perfringens* 6.5 °C; 4, includes flu-like disease, septicaemia, urethritis and abortion
*, chicken meat; **, fish

The major problem with MAP is the potential temperature abuse either during distribution or by mis-use by the consumer. Silliker and Wolfe (1980) studied MAP ground beef stored at 1 °C followed by temperature abuse (Table 1.14).

Table 1.14 The effect of temperature abuse of MAP ground beef on micro-organisms

ATMOSPHERE*	ORGANISM AND TEMPERATURE							
	Salmonella		*Staphylococcus*		Enterococci		*Clostridium sporogenes*	
	10 °C	20 °C	10 °C	20 °C	10 °C	20 °C	10 °C	20 °C
Air	+**	+	=	+	+	+	=	+
CO_2	+	+	-	+	=	+	-	-
CO_2+CO	=	+	-	+	=	+	-	-
CO_2 then air	+	+	-	+	+	+	-	=

All samples were stored at 1 °C for 4 days prior to temperature abuse

* CO_2 = 25%O_2 + 60%CO_2 + 15%N_2
CO_2 +CO = 25%O_2 + 60%CO_2 + 14%N_2 + 1%CO

** + >0.5log increase in growth
= <0.5log difference in growth
- >0.5log decrease in growth

Adapted from Silliker and Wolfe (1980)

The potential for growth of these pathogens in MAP meat packs must be properly evaluated with the presence of the background flora and in a variety of atmospheres. The relationship of growth of pathogens with the occurrence of different members of spoilage flora must be elucidated. Thus a thorough study of the species of bacteria present on MAP meats is needed.

CHAPTER 2

MICROBIAL ASSOCIATIONS ON MEAT

INTRODUCTION

Particular metabolic activities of various species of bacteria are responsible for the microbial spoilage of chilled foods. As the proportions of individual species or genera in a microbial consortium on meats vary considerably, selective media are commonly used to determine the actual contribution of particular micro-organisms to an association. In an ideal situation the antimicrobial agent(s) in a selective medium ought to allow growth of the desired micro-organism(s) without interference from others. The latter will grow on a selective medium if the concentration or balance of antimicrobial factors is sub-optimal. The use of large amounts of inhibitory agents may well curtail growth not only of unwanted organisms but of the desired ones also. In many situations in the food industry stressed or injured cells may not be recovered if the selectivity of a medium is too stringent. Streptomycin-thallous-acetate-actidione medium (Gardner, 1966) for *Brochothrix thermosphacta* is an example (Skovgaard, 1985). Indeed, it has been shown that a proportion of this species in British fresh sausage fails to grow on this medium but do so on plate count agar (Brown, 1977). These attributes of selective media must be borne in mind in any analyses of microbial associations on meat.

Two approaches have been taken to identify important micro-organisms in the spoilage of modified atmosphere packaged meats (Table 2.1). Many of the early studies used general purpose media (such as plate count agar) to enumerate the total aerobic population. Strains isolated from such a medium were characterised in order to determine the dominant species of a consortium. Numerically minor groups of an association would not be detected in this instance even though their metabolic attributes might well contribute to spoilage. In other cases selective media were used to determine the relative proportions of different bacterial groups - or yeasts for that matter (Tudor, 1989) - within a population. In general, the species isolated in the latter approach were not identified. Thus numerically important groups were highlighted but the dominant species not determined. Both approaches have shown that Gram positive bacteria generally become numerically dominant on MAP meats (e.g. Egan, 1983; Gill, 1983). This contrasts with the spoilage of aerobically stored chilled meats in which Gram negative (frequently pseudomonads) organisms have a dominant role (e.g. Ingram and Dainty, 1971; Egan *et al.*, 1991).

Lactobacillus, Leuconostoc, Carnobacterium, Lactococcus and *Pediococcus,* together with the catalase-positive *Brochothrix* are the dominant Gram positive bacteria

Table 2.1 Selective media used in studies of modified atmosphere packaged meats

Authors	Media used for enumeration of bacterial groups[a]					Further examination
	TAC[b]	LAB	Ps	Ent	Broch.	
Pierson *et al.*, 1970	APT	ATJS	PsF	EMB	STAA	✓[c]
Huffman *et al.*, 1975	PCA	TJA				
Sutherland *et al.*, 1975	TGYA	MRS	MGV	VRB	STAA	✓
Seiderman *et al.*, 1976	PCA	MRS	PsF			✓
Silliker *et al.*, 1977	PCA	ROG	MacC	MacC	[d]	
Christopher *et al.*, 1979a	PCA	MRS				✓
Christopher *et al.*, 1979b	PCA	MRS				✓
Enfors *et al.*, 1979	TGE	AcA		VRB		✓
Newton and Rigg, 1979	APT				STAA	
Erichsen and Molin, 1981	TGE	MRS			STAA	✓
Hanna *et al.*, 1981	TSA					✓
Egan and Shay, 1982	TSA	AcA		VRB		✓
Johnston *et al.*, 1982		APT	MacC	MacC		✓
Vanderzant *et al.*, 1982	TSA					✓
Blickstad and Molin, 1983	TGE	AcA		VRBD	STAA	✓
Gill and Penney, 1985	PCA					✓
Gill and Penney, 1986	PCA					✓
Gill and Penney, 1988	PCA					✓
Kalchayand *et al.*, 1989	PCA	APT				
Seman *et al.*, 1989	PCA	ROG				✓
Kleinlein and Untermann, 1990	PCA		Ps-Aer	VRBD		
Taylor *et al.*, 1990	PCA	MRS	CFC	VRBG	STAA	
Grant and Patterson, 1991b	GNA BHIYE					✓
McMullen and Stiles, 1991	PCA	MRS	CFC	VRBG	STAA	
Ordóñez *et al.*, 1991	PCA					✓
Rousset and Rennerre, 1991	PCA	MRS	CFC	VRBD	STAA	
Lambert *et al.*, 1992	PCA	MRS		VRBG		
Buys *et al.*, 1993	Std 1	MRS	Kiel	DHL		
Greer *et al.*, 1993		MRS	CFC	VRBG	STAA	

a TAC total aerobic count; LAB lactic acid bacteria; Ps *Pseudomonas* spp.; Ent Enterobacteriaceae; Broch. *Brochothrix thermosphacta*

b AcA, Acetate agar; APT, All purpose tween; ATJS, - cited to **Jaye, M.; Kittaka, R.S. and Ordal, Z.J.** (1962) The effect of temperature and packaging material on the storage and bacterial flora of ground beef. *Food Technology,* **16: 95 - 98**; BHIYE, Brain heart infusion yeast extract; CFC, Cephaloridine-Fucidin-Cetrimide; DHL, - cited to **Sakazaki, R.; Nanioka, S. and Osada, A.** (1960) A problem on the pathogenic role of *Citrobacter* of enteric bacteria. *Jap. J. Ex. Med.*, **30**: 13-22; EMB, Eosin methylene blue; GNA, Glucose nutrient agar (NA + 0.5% glucose); Kiel, cited to **Kleinlein, G.** (1971) Die isoliering und differenzierung von Pseudomonaden aus lebensmiteln. *Arch. Lebensmittelhyg.*, **22**: 29-37; MacC, MacConkey; MRS, de Man, Rogosa, Sharpe; MGV, Masuvosky, Goldburn and Voss; PCA, Plate count agar; PsF, Pseudomonas agar for fluorescent pseudomonads (King *et al.*, 1954); PsAer, - cited to **Kielwein, G.** (1969) Ein Nährboden zur selektiven Züchtung von Pseudomonaden und Aeromonaden. *Arch. Lebensmittelhyg.*, **20**: 131-133; Std 1, ROG, Rogosa; STAA, Streptomycin, thallous-acetate, actidione; TGYA, Tryptone glucose yeast extract (=PCA); TGE, Tryptone glucose extract; TJA, Tomato juice agar; TSA, Tryptone soya agar; VRB, Violet red bile; VRBD, Violet red bile dextrose; VRBG, Violet red bile glucose;

c Isolation and identification of bacteria from TAC or selective media

d Gram positive bacteria enumerated on ABA (Azide blood agar)

in MAP meats. The selective medium for *Br. thermosphacta* - streptomycin-thallous-acetate-actidione medium (Gardner, 1966) - has met with general acceptance because of its reliability in the isolation of this organism even though its selectivity may be too stringent. Many selective media have been developed for the enumeration of

particular genera of lactic acid bacteria, a heterogeneous group of organisms having relatively fastidious growth requirements. Even though a variety of selective media is available, the enumeration of all members of this group without the growth of other organisms remains a problem. Baird and Patterson (1980) compared the efficacy of several selective, elective - nutrient status enables good growth of all or special groups (Reuter, 1985) - and non-selective media. The most selective media were MRS at pH 5.5, NAP and Rogosa agar. None of these, however, is suitable for the enumeration of *Carnobacterium* spp. (Collins *et al.*, 1987) which fail to grow at low pH (*ca* 5.5) levels, particularly in the presence of acetate. Indeed, Hitchener *et al.* (1982) indicated that in a number of studies the bacterial counts on MRS were 75 - 100% of those on non-selective media.

As the present study was part of a collaborative venture, media suitable for the enumeration of bacteria likely to be present on modified atmosphere packaged meats were selected at the outset from reports in the literature (Table 2.1). Experience showed that, although the adopted media were in common use, growth of bacterial groups other than those sought occurred in some cases. In one instance this was ameliorated by amendment of a medium, e.g. CFC (insert to page 45).

A study was done initially to assess inter-laboratory variation of results. The changes in the microbial population developing on MAP meats were determined. This highlighted the inadequacy of some of the selective media chosen for this study. Consequently further work was done to determine the extent of this problem. In practice the "contamination" of certain selective media was such a common feature that modifications were attempted. Much of the work presented in this Chapter was done concurrently with the identification of organisms selected from particular trials (Chapters 4-6).

PART 1 GENERAL TRENDS IN THE MICROBIAL ASSOCIATIONS DEVELOPING ON MAP MEAT

Introduction

The initial experiment was done to validate the adopted methods for the analysis of the microflora on MAP beef steaks, to study different selective media (Table 2.2) and to determine the inter-laboratory variation of the results. The beef steaks packed at Leatherhead Food Research Association (LFRA) were stored in a cold room (approximately 4 °C), and similar techniques used to analyse these at both LFRA and Bath University (BU).

Materials and Methods

Steaks weighing 53 ± 3g were vacuum packed in Suprovac 90 vacuum pouches (90 µm thick, Kempner Ltd., Perry Road, Industrial Estate East, Witham, Essex. CM8 3TY). The gas permeability of the pouches (given in transmission rates (TR) was OTR (oxygen) *ca* 25, CDTR (carbon dioxide) *ca* 90 and NTR (nitrogen) *ca* 6 $m^3m^{-2}day^{-1}bar^{-1}$ at 20 °C and 50% relative humidity (RH). The permeability of water vapour was *ca.* 1.1 $gm^{-2}d^{-1}$ at 23 °C and 85% RH. The settings for the Suprovac 180 vacuum packer (Berkel Ltd., 72, Cobden Street, Leicester. LE1 2LE) were: full vacuum, vacuum time setting of 8 and seal temperature setting of 9. Steaks in high density polyethylene trays (Dynopack, Orion House, Calleva Industrial Park, Aldermaston, Reading, Berkshire. RG7 4QW) were packed in modified atmospheres using a Mecapac M500 machine (Swissvac, Unit A, Marish Wharf, St. Mary's Road, Langley, Berkshire. SL3 6DA) with a Kempner Suprovac 90 top web (permeability data as for vacuum pouches). The pre-mixed food-grade gases (80% O_2 + 20% CO_2, 50% N_2 + 50% CO_2 or 100% CO_2) were supplied by BOC (Unit 1C, Roxborough Way, Maidenhead, Berkshire. SL6 3UD). Approximately 2 litres of gas were added per kg of meat.

STORAGE

Packs were stored at 0 or 5 °C in chilled incubators and analysed at intervals up to 46 days.

MICROBIOLOGICAL EVALUATION

At each sampling time, the outer surface of a pack was swabbed with 70% (v/v) ethanol to reduce contamination. After opening with sterile (flamed) equipment, the steak was chopped into small pieces some of which (10 g) were homogenised in a Colworth Stomacher (A.J. Seward Ltd., UAC House, Blackfriars Road, London, SE1) for 60 s with 90 ml maximal recovery diluent (MRD, Lab M). The homogenate was used to prepare a decimal dilution series - 9ml MRD + 1ml of macerate. The appropriate dilutions were used with spread or pour plates (Table 2.2). Duplicate plates of each medium were used. The pH of the initial homogenate was measured using a Pye Unicam (PW 9410) pH meter.

ASSESSMENT OF MEDIA SELECTIVITY

Colonies from the selective media were purified by streaking on nutrient agar (Lab M) for CFC and VRBG isolates, Whittenbury's basal medium with glucose (0.5% yeast

Table 2.2 Selective media used in microbiological testing - methods of use and incubation conditions

Medium[a]	Method[b]	Incubation °C	Incubation (d)	conditions atmosphere[c]	Bacteria sought
PCA[d]	Spread	25	3	Aer	Total aerobic count
PCA	Spread	25	3	An	Lactic acid bacteria
CFC	Spread	25	3	Aer	*Pseudomonas* spp.
STAA	Spread	25	2	Aer	*Brochothrix thermosphacta*
RBC	Spread	25	5	Aer	Yeasts
MRS	Pour	25	3	Aer	*Lactobacillus* spp.
VRBG	Pour	30	2	Aer	Enterobacteriaceae

a LFRA used All Purpose Tween (APT, Difco) instead of PCA. No RBC or MRS was used in their study.
b Spread plates were inoculated with 100 µl and pour plates with 1 ml of the appropriate dilution
c Aer aerobic; An anaerobic (Oxoid gas generating kit)
d PCA Plate count agar (Lab M); CFC Cephaloridine, Fucidin, Cetrimide (Lab M); STAA Streptomycin Thallous Acetate Actidione (Oxoid); RBC Rose bengal chloramphenicol (Lab M); MRS de Man, Rogosa, Sharpe (Lab M); VRBG Violet Red Bile Glucose (Oxoid)

extract, 0.5% lab lemco, 0.5% bacteriological peptone, 0.5% glucose, 0.05% Tween 80 and 1.2% tissue culture agar) for lactic acid bacteria and *Br. thermosphacta* and on RBC for yeasts. The purified bacteria were Gram stained and the cell morphology of yeast was studied. Pure cultures of bacteria isolated from CFC and VRBG were tested for oxidase

reaction and mode of glucose metabolism. The oxidase reaction was determined with cells transferred with a wooden stick to filter paper soaked in N,N,N,N, tetramethyl-*p*-phenylenediamine (Kovac's reagent as described in Harrigan and McCance, 1976). A purple colour was recorded as positive. The Hugh and Leifson test (Harrigan and McCance, 1976) for determining the mode of metabolism of glucose was done. Strains that were oxidase positive and used glucose oxidatively were deemed to be pseudomonads. Oxidase negative, fermentative organisms were assumed to be members of the Enterobacteriaceae.

Results

The microbial analysis for the initial trial at Bath University is presented in Figures 2.1 and 2.2. In general the results were similar to those at Leatherhead (data not shown), the exception being only colony size. It was very much smaller on anaerobic PCA plates (BU) *vis à vis* APT (LFRA). Only one pack was tested at each sample time as the preliminary nature of the experiment did not warrant the use of large amounts of meat. The numbers of bacteria present initially were higher than had been anticipated. In the UK, levels of 10^4 - 10^5 cfu/g are common on red meats taken from an abattoir. In this study populations of $>10^6$ cfu/g were present at the time of packaging. The majority of the population was lactic acid bacteria, again a most unusual feature of fresh meat.

The colony types growing on selective media were examined to determine the efficacy of the selective media. The vast majority of the isolates from RBC proved to be yeasts. Gram positive rods were a dominant feature of STAA and MRS. *Pseudomonas* spp. and Enterobacteriaceae were minor portions of the meat microflora. This may in part be the reason for the selectivity of CFC and VRBG being compromised under the test conditions. Analysis showed that only 73% of the isolates from CFC were members of the genus *Pseudomonas*. In contrast, 76% of strains from VRBG were Enterobacteriaceae. Obviously when these two media were used for MAP meats, their selectivity was not totally reliable.

The numbers of lactic acid bacteria on meat remained static or decreased slightly during storage at 0 °C. The other groups of micro-organisms tended to survive but not grow in the low CO_2 atmospheres (80% O_2 + 20% CO_2 and vacuum packs). Their numbers

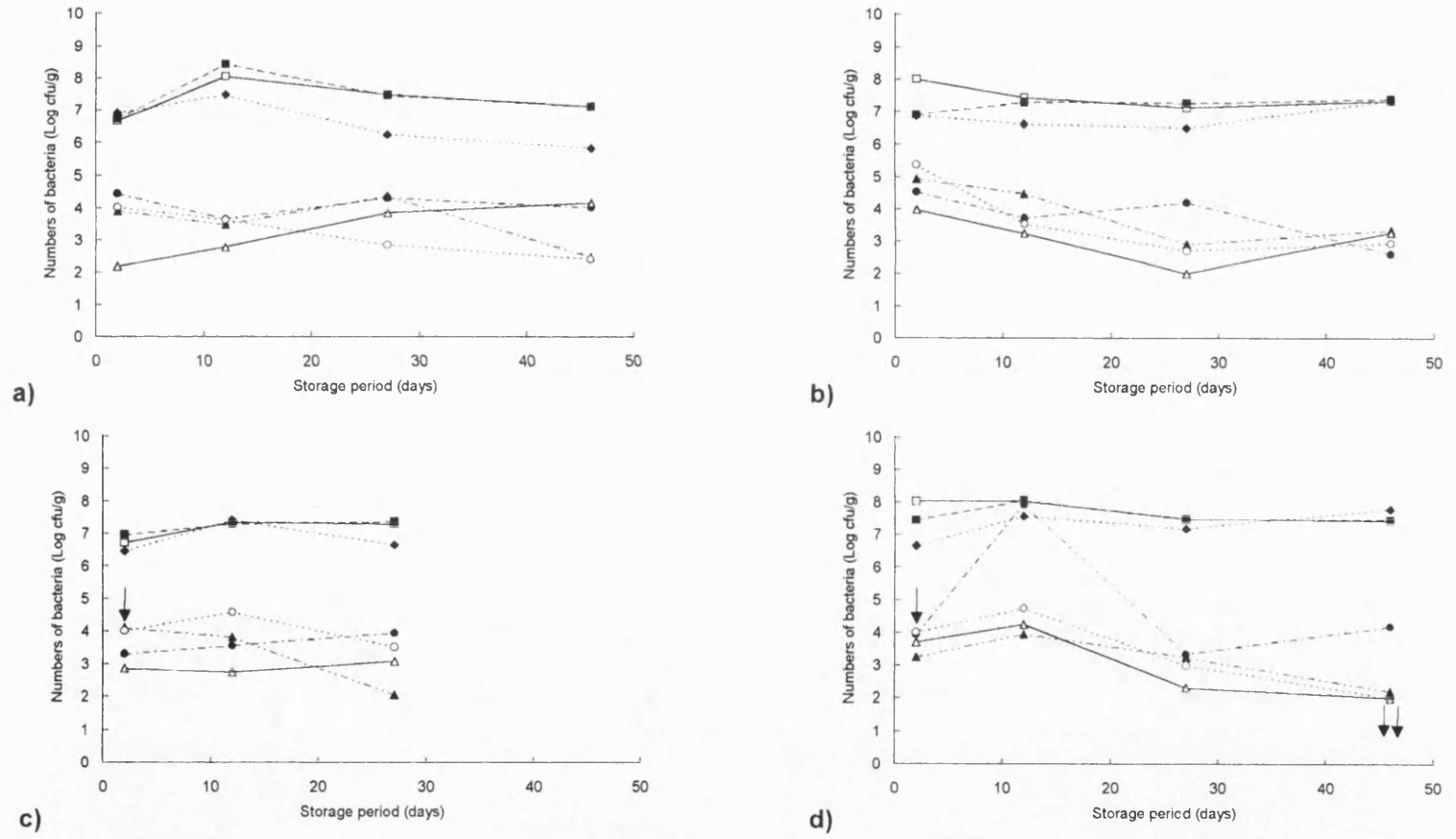


Figure 2.1 **Microbial flora developing on beef steaks stored at 0 °C in a) vacuum pack b) 50% N_2 + 50% CO_2 c) 80% O_2 + 20% CO_2 or d) 100% CO_2**

□ Total aerobic count; ■ Total anaerobic count; ◆ lactobacilli; ○ *Brochothrix thermosphacta*; ● *Pseudomonas* spp.; ▲ Enterobacteriaceae; △ yeasts

Arrow indicates numbers were below the limit of detection

See Table 2.2 for media

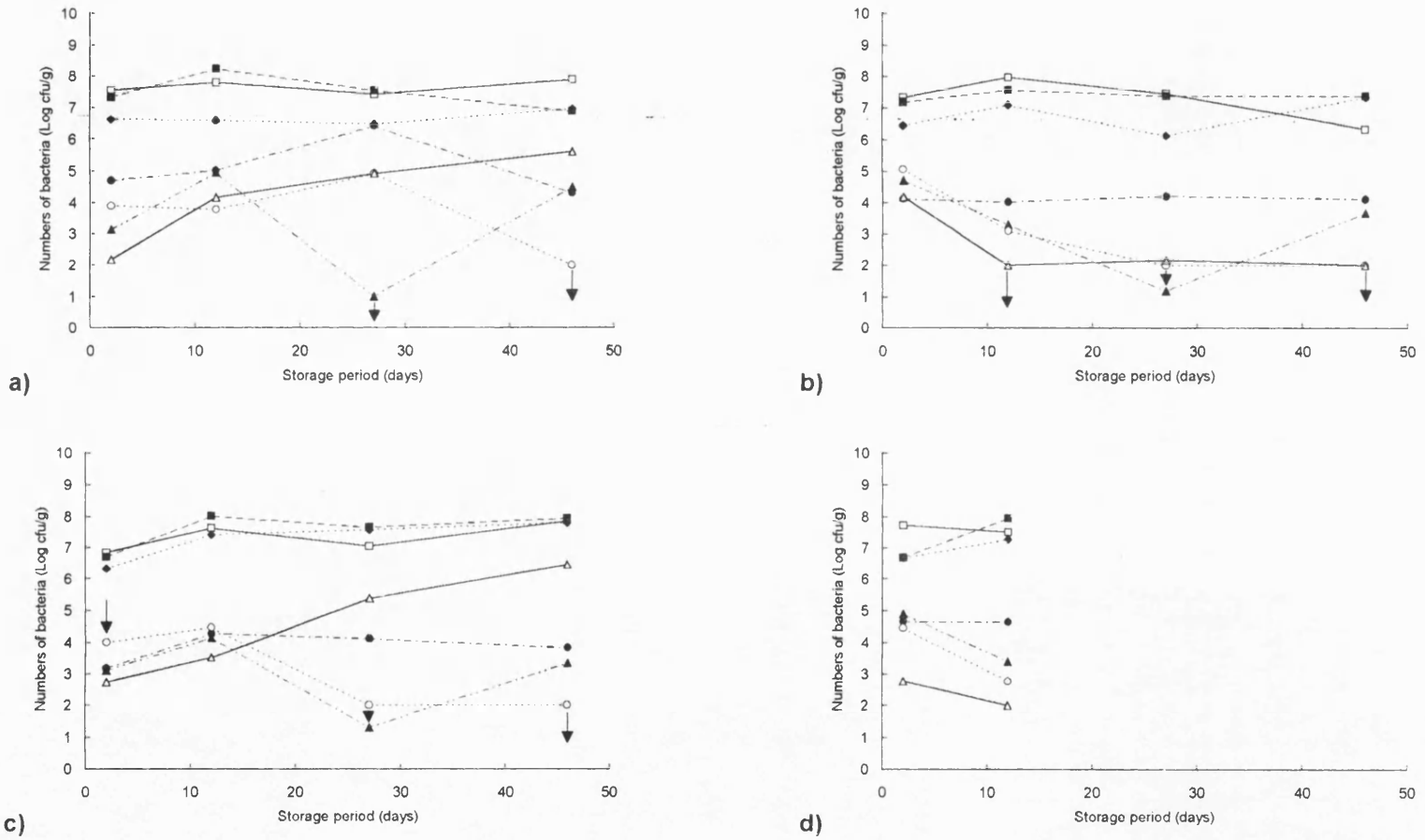


Figure 2.2 **Microbial flora developing on beef steaks stored at 0 °C in a) vacuum pack b) 50% N_2 + 50% CO_2 c) 80% O_2 + 20% CO_2 or d) 100% CO_2**

□ Total aerobic count; ■ Total anaerobic count; ◆ lactobacilli; ○ *Brochothrix thermosphacta*; ● *Pseudomonas* spp.; ▲ Enterobacteriaceae; △ yeasts

Arrow indicates numbers were below the limit of detection

See Table 2.2 for media

decreased during storage in high (>50%) CO_2 atmospheres. An unexpected feature was noted after 12 days storage in 100% CO_2. The numbers of pseudomonads were log_{10} 3.94 at day 2, 7.93 on day 12 and 3.34 on day 27. This was almost certainly due to a leaking pack being analysed on day 12. Aerobic pseudomonads would not be expected to increase significantly in numbers in the high CO_2 atmosphere, whereas they are well known to dominate the consortium on aerobically stored meats (e.g. Pierson *et al.*, 1970; Shelef, 1981). Samples taken at other times did not show this trend.

Trends similar to those described above were noted also with storage at 5 °C. Again there were obvious imperfectly sealed packs of meat in CO_2 atmospheres. The meat in such packs was overtly spoiled at the time of analysis. These were not tested further. There was a slight increase in numbers of yeasts on the beef steaks packed in 80% O_2+ 20% CO_2 and VP but their growth was inhibited in atmospheres containing higher proportions (≥ 50%) of CO_2. The numbers of *Brochothrix thermosphacta* decreased throughout storage in all atmospheres.

Discussion

The results from the two laboratories (Bath University and LFRA) would be expected to be similar because the same source of meat and storage conditions were used. Even with the use of different media, common trends were evident in the two trials. The APT medium used at Leatherhead gave similar results to those obtained on PCA at Bath, e.g. a dominance of lactic acid bacteria. The colonies forming on APT were larger than those on PCA, due presumably to the higher nutrient status of the former. APT was chosen for further work.

Lactic acid bacteria dominated the microflora. The numbers of LAB on MRS were generally one log lower than those on PCA. The difference between the selective and general purpose medium may well be due to the failure of some LAB strains to grow on the selective medium. This trend has been noted by others (e.g. Hitchener *et al.*, 1982). As noted in the Introduction to this section *Carnobacterium* spp. fail to grow in the presence of acetate at a pH < 5.5 (Collins *et al.*, 1987). Members of this genus have been isolated from meats on MRS at pH 6 - 6.5 and on a non-selective media also, e.g. tryptone glucose extract agar (Borch and Molin, 1988). It was decided to use APT with incubation in an atmosphere enriched with CO_2 in subsequent studies.

The prevalence of lactic acid bacteria at the time of packing was associated with abattoir practice in the sense that the meat had been vacuum packed before delivery to LFRA. The selective pressures (high CO_2/low O_2) of this form of packaging had obviously favoured the growth of these organisms. The microbial analysis of the MAP beef steaks during storage also endorsed the view of others (e.g. Reuter, 1985; Taylor *et al.*, 1990), namely that high pCO_2 (e.g. 50% N_2 + 50% CO_2 and 100% CO_2 at 0 °C) favours the growth of LAB.

This preliminary experiment showed that the adopted selective media were in general suitable, even though there were problems with CFC and VRBG.

PART 2 MEDIA SELECTIVITY

Introduction

As outlined above, the media used for the enumeration of the microbial flora from MAP meats were generally suitable. A medium for the enumeration and isolation of all members of the lactic acid bacteria, numerically dominant in this environment, without the growth of other micro-organisms would be ideal. Many have been devised for members of the LAB; Section 1 outlines a study to determine the most suitable medium for MAP meat.

Pseudomonas and Enterobacteriaceae, generally present as a minor portion of the flora in MAP meats, are enumerated with selective media. The pseudomonad medium, CFC (Mead and Adams, 1977) was devised for meats and poultry and tested with aerobically stored meats, an environment in which pseudomonads are normally predominant. Thus, the medium has not been tested previously under such stringent conditions. The violet red bile glucose medium (VRBG) was designed for general food and water use rather than for meats. It should, therefore, be tested with the latter to determine any short-comings.

SECTION 1 LACTIC ACID BACTERIA

Introduction

The efficacy of a selective medium is linked inextricably to the environment or foodstuff for which it was designed. Thus with LAB, the majority of the widely used selective media are derived from those devised for studies of silage (Keddie, 1951) or the microflora of the mouth (Rogosa *et al.*, 1951). A major derivation - and one adopted by many meat microbiologists - is MRS. It was based on the original paper by Rogosa and his colleagues (de Man *et al.*, 1960). Nitrite-actidione-polymyxin (NAP) medium was designed specifically for enumeration of lactic acid bacteria from foods (Davidson and Cronin, 1973). It has a low pH and is therefore not entirely suitable for use with foods likely to contain *Carnobacterium* spp. or other non-aciduric LAB. The relative proportion of bacteria present in the microbial association will also determine the effectiveness of selectivity. Thus, different selective and elective media for lactic acid bacteria were incubated in different atmospheres (aerobic and anaerobic) and compared to determine their efficiency with MAP meats.

Materials and Methods

Coarsely minced beef shin was vacuum packed (100 g portions) at the time of purchase from a local butcher. The packs were stored at 5 °C. When sampling, the surface of the pack was wiped with 70% (v/v) ethanol to reduce contamination. Ten g were removed aseptically and homogenised with 90 ml MRD (Lab M) for 60 s in a Colworth Stomacher. A decimal dilution series was made and appropriate dilutions were plated onto the different media (Table 2.3). These were incubated in aerobic or anaerobic (using gas generating packs, Oxoid) atmospheres. Media for the enumeration of bacterial groups likely to be found on MAP meats were included along with media for the lactic acid bacteria. There were two objectives to this study, to determine patterns in population change and to compare selective and elective media for lactic acid bacteria.

Table 2.3 Media used in a trial intended to optimise the isolation of lactic acid bacteria from MAP meats

Medium[a]	Inoculation Method[b]	Incubation conditions: Atmosphere[c]	Temperature (°C)	Time (h)
For lactic acid bacteria:				
APT	Spread	Aerobic Anaerobic	25	72
MRS[d]	Pour	Aerobic Anaerobic	25	72
YES	Pour	Aerobic Anaerobic	25	72
ROG	Pour	Aerobic Anaerobic	25	72
TJA	Pour	Aerobic Anaerobic	25	72
NAP	Pour	Aerobic Anaerobic	25	72
For other microbial groups:				
CFC	Spread	Aerobic	25	48
STAA	Spread	Aerobic	25	48
VRBG	Pour	Aerobic	30	48

a APT, All Purpose Tween (Difco); MRS, de Man, Rogosa, Sharpe (Lab M); YES, Yeast extract plus sucrose (made up from Lab M and BDH components); ROG, Rogosa (made up from Lab M and BDH components); TJA, Tomato juice agar (made up from Lab M and BDH components); NAP, Nitrite actidione polymyxin (made up from Lab M and BDH components); CFC, Cephaloridine fucidin cetrimide (Lab M); STAA, Streptomycin thallous acetate actidione agar (Lab M); VRBG, Violet red bile glucose agar (Lab M)

b 100 µl of the appropriate dilutions were spread onto plates, 1 ml being used for pour plates

c Anaerobic gas generating system (Oxoid) was used to create anaerobic conditions

d MRS and Modified MRS (MRS + 0.1% w/w cysteine hydrochloride and 0.2% potassium sorbate pH 5.7 - von Holy and Cloete, 1992) were used after two weeks of storage.

Results

The sizes of the microbial populations developing on vacuum packaged minced beef shin are given in Figure 2.3. *Pseudomonas* spp. were dominant initially but their

numbers declined during storage. Lactic acid bacteria (as enumerated on APT incubated anaerobically) became the most numerous group within the first two weeks of storage. The populations of *Brochothrix thermosphacta* remained static throughout storage. After an initial increase from *ca* 10^2 - 10^4 cfu/g in the first two weeks, the numbers of Enterobacteriaceae did not change significantly during the eight week study.

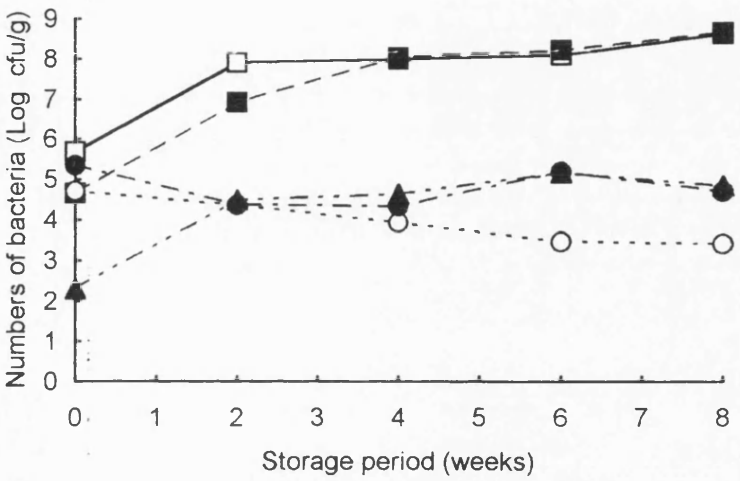


Figure 2.3 **Microbial flora developing on vacuum packaged minced beef stored at 5 °C.** □ Total aerobic count (APT); ■ Total anaerobic count (APT); ○ *Brochothrix thermosphacta* (STAA); ● *Pseudomonas* spp. (CFC); ▲ Enterobacteriaceae (VRBG)

Initially the numbers calculated from the elective media (APT, YES, TJA) were *ca* 10^5 log cfu/g (Figure 2.4), whilst those of the selective media (MRS and ROG) were significantly less (10^2-10^3 log cfu/g). Modified MRS (see Table 2.3) was not used until the meat samples had been stored for two weeks. After this time, the numbers of bacteria on selective and elective media were comparable. This was to be expected because the initial population on meat was dominated by pseudomonads, with the lactic acid bacteria becoming prevalent only during the first two weeks of storage. Thus initially, the elective media supported growth of micro-organisms (e.g. pseudomonads) other than LAB but with time the counts of the latter were equivalent to those on the selective media. The selectivity of Rogosa's medium tended to be more pronounced than that of the MRS medium. This may be due to the growth of non-lactic acid bacteria on MRS or because some species within the LAB group were unable to grow on ROG. A similar argument can be used for the differences in counts with elective *vis à vis* selective media.

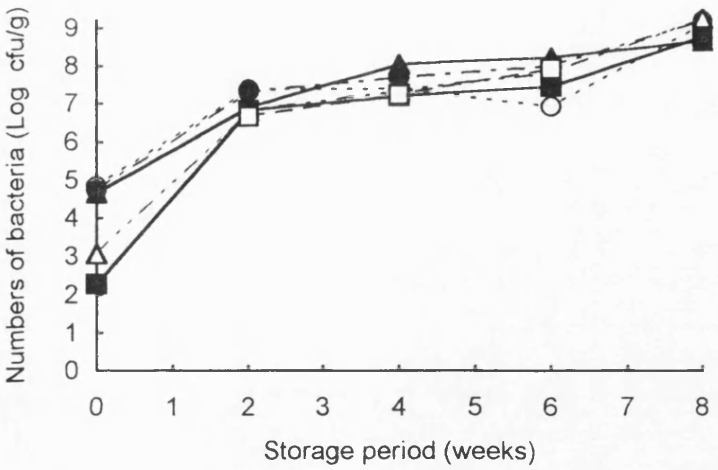


Figure 2.4 **Lactic acid bacteria developing on vacuum packaged minced beef stored at 5 °C.** Enumerated on ▲ APT incubated anaerobically; ❍ Yeast extract sucrose; ● Tomato juice; △ MRS; ■ Rogosa; □ modified MRS

The atmosphere of incubation affected the results. Figure 2.5 gives the data for the initial sample only (day 0), those for other storage times were similar with aerobic or anaerobic incubation. Rogosa's medium proved to be an exception in that the numbers of organisms on the medium incubated anaerobically were larger than those grown aerobically. All the other media gave larger numbers on the plates incubated aerobically. The largest difference between the counts obtained with aerobic and anaerobic incubation was seen with APT. This highly nutritious medium allowed good growth of the fastidious lactic acid bacteria.

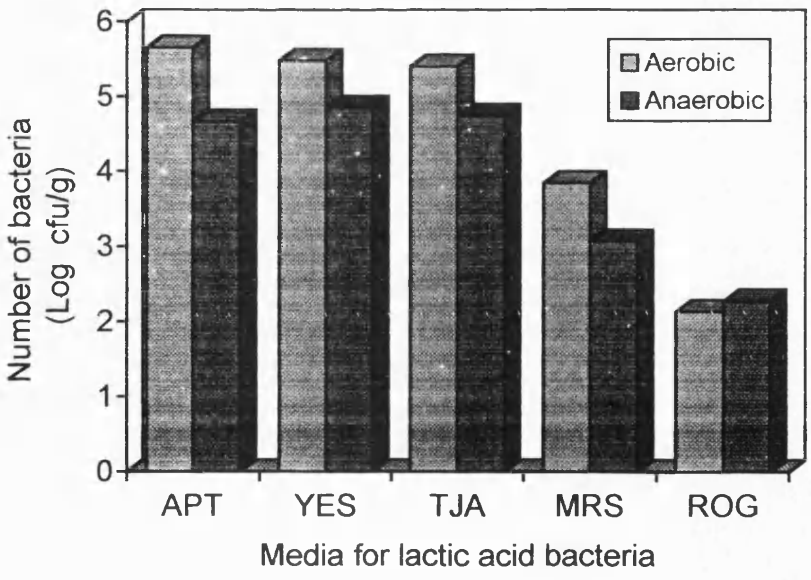


Figure 2.5 **The influence of incubation atmosphere on the selectivity of media intended for the isolation of lactic acid bacteria from MAP meat.** As enumerated on All Purpose Tween (APT); Yeast extract sucrose (YES); Tomato juice agar (TJA); de Man, Rogosa, Sharpe (MRS); Rogosa (ROG)

Another experiment was made with vacuum packaged minced beef (Figure 2.6). It included MRS and an additional selective medium, NAP (Davidson and Cronin, 1973). The development of the microbial association was the same as that described above, lactic acid bacteria became dominant, whilst the numbers of pseudomonads, Enterobacteriaceae and *Brochothrix thermosphacta* did not increase significantly during storage. Initially MRS gave a count *ca* 1 $\log_{10}$ cfu/g lower than that on APT. The number of colonies on MRS medium and the total aerobic count (APT) were of the same magnitude, however, after the second week of storage. The NAP medium gave very low counts initially (<10^2 cfu/g) and these were invariably lower than those on the MRS medium throughout storage.

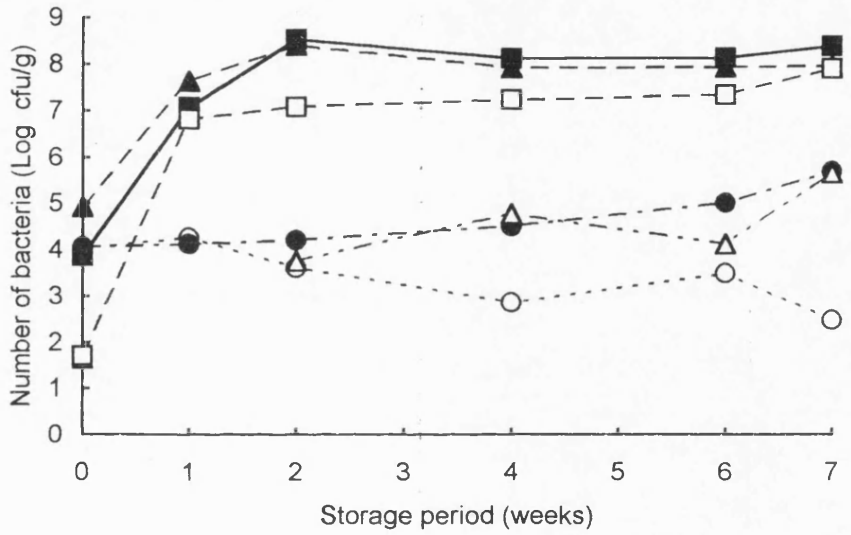


Figure 2.6 **The microbial flora developing on vacuum packaged minced beef stored at 5 °C.** ▲ APT incubated aerobically; ❍ STAA (*Brochothrix thermosphacta*); ● CFC (*Pseudomonas* spp.); △ VRBG (Enterobacteriaceae); ■ MRS (lactic acid bacteria); □ NAP (lactic acid bacteria)
Data for VRBG at week one was not given because of contamination on the plates.

When the identity of isolates from both elective and selective media were considered (see Chapter 4 and Appendix 2 for identification details), it was evident that *Lact. sake* was the predominant organism (group 1, Figure 2.7). This dominance was reflected in the results obtained with all media other than YES. The latter favoured the growth of the organisms for which it was designed, *Leuconostoc* spp. It needs to be stressed also that the proportion of *Lact. sake* on ROG exceeded those on all other media, a good example of the bias that can occur from the use of a highly selective medium. In practice though, TJA, APT, MRS and NAP could all be used for study of LAB of chill-stored meats.

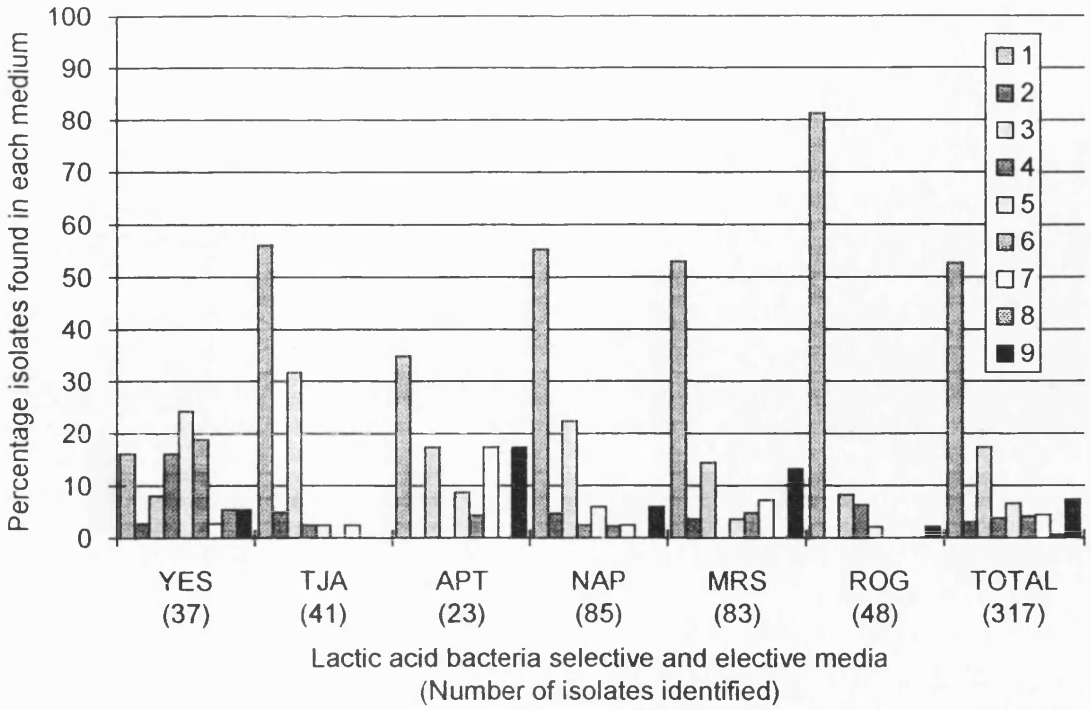


Figure 2.7 **Differences in the selectivity of media for the enumeration of lactic acid bacteria.**

Groups 1 and 2, *Lactobacillus sake/curvatus*; 3 and 4, *Carnobacterium divergens*; 5 and 6, *Leuconostoc carnosum.*; 7, *Leuconostoc gelidum.*; 8, *Leuconostoc mesenteroides.*; 9, Unidentified lactic acid bacteria

YES, Yeast Extract Sucrose; TJA, Tomato Juice Agar; APT, All Purpose Tween; NAP, Nitrite-Actidione-Polymyxin; MRS, de Man, Rogosa, Sharpe; ROG, Rogosa

Discussion

As previous reviews have stressed, the media recommended for the selection and enumeration of lactic acid bacteria are not entirely satisfactory (Baird and Patterson, 1980; Reuter, 1985; Holzapfel, 1992). Each medium was designed for a particular purpose; thus some variation would be anticipated in the species selected. Yeast extract sucrose (YES) is an elective medium, which is used to enumerate the characteristic slimy colonies of dextran-producing leuconostocs. All Purpose Tween - APT - was proposed by Evans and Niven (1951) for the enhanced growth of *Lactobacillus viridescens* but has been used widely as an elective medium for lactic acid bacteria (Baird and Patterson, 1980). Tomato juice agar - TJA (Briggs, 1953) was used to enumerate lactobacilli from milk products, with de Man, Rogosa, Sharpe - MRS (de Man *et al.*, 1960) medium being a defined version of the same medium. Neither is particularly selective. Rogosa - ROG - agar (Rogosa *et al.*, 1951) was designed to isolate selectively oral and faecal lactobacilli. The only medium which was specifically designed for the enumeration of lactic acid bacteria as a whole from foodstuffs

was Nitrite-Actidione-Polymyxin (NAP) medium of Davidson and Cronin (1973). The results from this study illustrate the selective nature of ROG in particular and MRS and NAP to a lesser extent. Thus, the choice of media for the enumeration of lactic acid bacteria must be a compromise. As the species important in MAP beef steaks were studied in the course of present work, an elective (APT) rather than selective medium (e.g. MRS) was chosen. Anaerobic incubation promoted growth of the lactic acid bacteria relative to that of aerobic organisms. There were still problems, however, with the growth of some organisms at the beginning of storage. *Brochothrix thermosphacta* grew on APT and, when present in high numbers in the meat, affected the enumeration of LAB.

SECTION 2 SELECTIVITY OF MEDIA FOR *PSEUDOMONAS* SPP. (CFC) AND ENTEROBACTERIACEAE (VRBG)

Introduction

Growth of members of the family Enterobacteriaceae on CFC and of pseudomonads on VRBG was noted in initial experiments. The selectivity of the media was checked using packs of modified atmosphere packaged meats. The recommended incubation temperature for VRBG is 37 °C. In the planning of the FLAIR programme, 30 °C was adopted in order to allow enumeration of psychrotrophic strains which may have optimum growth temperatures less than those of mesophiles. Three temperatures were tested in the present study to establish the optimum temperature for the enumeration of pseudomonads and Enterobacteriaceae.

Gram positive bacteria, particularly enterococci, form small colonies in VRBG agar. The manufacturer's instructions recommend that only colonies surrounded by precipitated bile ought be counted. The colonies growing in pour plates of VRBG varied in size. Consequently large and small colonies - regardless of the presence or otherwise of precipitate - were enumerated and each characterised separately.

Materials and Methods

Twenty g of minced lamb or beef (gas packed) from a local supermarket were homogenised in 180 ml of Maximal Recovery Diluent (MRD, Lab M) for 60 s in a Colworth Stomacher (A.J. Seward Ltd., UAC House, Blackfriars Road, London, SE1). A decimal dilution

series was prepared in MRD (1 ml of the homogenate + 9 ml MRD) and 0.1 ml of appropriate dilutions spread on the surface of the CFC (Lab M) and plate count agar (Lab M) to determine the size of total aerobic flora. One ml of appropriate dilutions was used in pour plates of VRBG. Duplicate plates were inoculated for each variable with incubation at 25, 30 or 37 °C. Colonies were enumerated after 48 h. Pure cultures were obtained from ten colonies growing on CFC and ten small and ten large ones on VRBG by streaking onto Nutrient agar (NA, Lab M).

Pure cultures were tentatively assigned to the genus *Pseudomonas* or the family Enterobacteriaceae on the basis of Gram and oxidase reaction and mode of glucose metabolism (described on pp. 38-39).

Results

In the majority of cases larger numbers of bacteria were isolated on CFC and VRBG with incubation at the lowest (25 °C) temperature (Figure 2.8). In practice the differences in counts, particularly for minced lamb, were less than one log cycle (taken to be within experimental variation). With beef the numbers of bacteria recovered with incubation of media at 37 °C were more than one log lower than those at 30 °C with PCA, CFC and for the large colonies in VRBG.

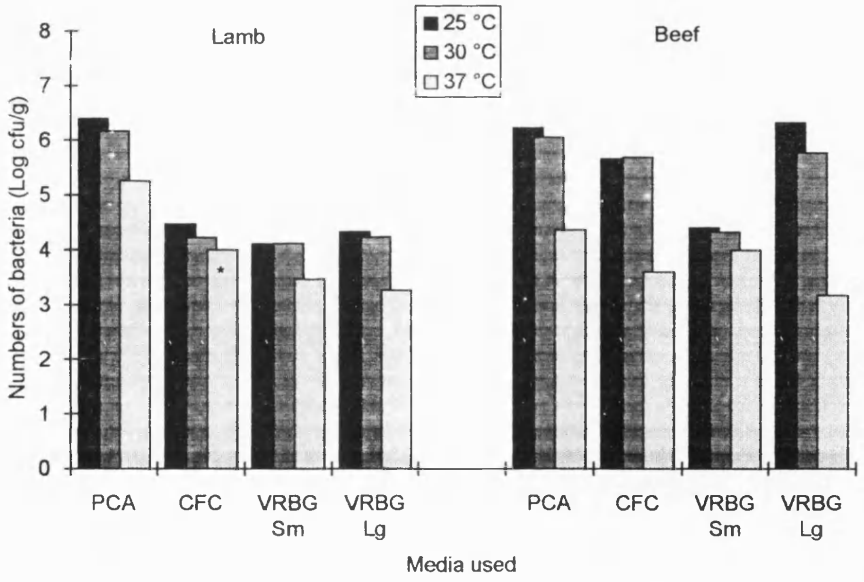


Figure 2.8 **Numbers of bacteria recovered from modified atmosphere packaged meats on different media at various temperatures**

* Numbers less than 10^4, below the limit of detection

Sm, Small colonies; Lg, Large colonies

In contrast to previous results (pp. 39) the cephaloridine-fucidin-cetrimide medium of Mead and Adams (1977) did not allow the growth of Enterobacteriaceae. In the initial analysis (pp. 37), plates were stored for various periods before isolation and purification of organisms. The growth of Enterobacteriaceae appeared to have occurred during the storage period. As judged by the negative oxidase reaction and fermentation of glucose, the large colonies on VRBG were all members of the family Enterobacteriaceae. With incubations at 25 and 30 °C, the small colonies on VRBG were assigned to *Pseudomonas.* At 37 °C the majority were assigned to Enterobacteriaceae (Table 2.4).

Table 2.4 Selectivity of CFC and VRBG incubated at different temperatures*

Medium (& colony size for VRBG)	Percent of positive isolations from media incubated at different temperatures (°C) e.g. % pseudomonads from CFC and Enterobacteriaceae from VRBG					
	Lamb			Beef		
	25	30	37	25	30	37
CFC	100[a]	100	N.D.[b]	100	100	100[c]
VRBG Small	0[d]	0[d]	70[e]	0[d]	0[d]	80[f]
VRBG Large	100	100	100	100	100	100

* Media were inoculated with bacteria from modified atmosphere packaged minced lamb and beef
a Ten isolates were tested unless otherwise stated
b N.D. No bacteria were present on CFC at this temperature
c Five isolates only were present on VRBG at 37 °C
d All isolates were pseudomonads
e Three isolates were *Aeromonas* (oxidase positive/fermentative/DNase positive)
f Two isolates were pseudomonads

Discussion

The two meat species (beef and lamb) showed different trends in relation to the isolation of pseudomonads and Enterobacteriaceae from CFC and VRBG respectively. The bacterial counts on beef showed larger differences at the three different temperatures than those on lamb. This effect may be related to the physiological characteristics of the meat. Beef normally has a pH of 5.5 - 5.75 whereas that of lamb is 5.75 - 6.0. At the lower pH of beef, cells may be stressed such that on transfer to a selective medium at the highest temperature, the synergistic action may cause cells to become non-culturable or to die.

As CFC did not support the growth of Enterobacteriaceae in the current experiment, it was used in further studies. Some pseudomonads grew on VRBG with incubation at 30 °C. Their colonies could be distinguished by their small size at this temperature - all the large colonies proved to be Enterobacteriaceae. Some pseudomonads grew on VRBG even

at 37 °C. This tended to inflate the counts on this medium. In practice VRBG at 30 °C was deemed to be generally suitable for analyses of the population of Enterobacteriaceae providing only the large colonies were enumerated.

PART 3 MODIFIED MEDIA

Introduction

In subsequent studies (see Chapters 4 & 5) growth of Enterobacteriaceae on CFC and *Pseudomonas* spp. on VRBG medium was noted. Consequently, these media were amended to allow differential counts to be made. The development of a selective-differential medium for the enumeration and identification of the three meat pseudomonad species is outlined. Each section is detailed separately.

MODIFIED CFC

See insert to this page (54).

Modified VRBG

MATERIALS AND METHODS

The Enterobacteriaceae medium, VRBG, was amended by the addition of 1% (w/v) L-arginine hydrochloride (Sigma) and 0.002% (w/v) Phenol Red (BDH). The composition (%w/v) of the final medium was: yeast extract 0.3, bacteriological peptone 0.7, sodium chloride 0.5, bile salts 0.5, crystal violet 0.0002, L-arginine hydrochloride 1.0, and phenol red 0.002. All constituents were from Lab M, Oxoid or BDH. The constituents were suspended in distilled water, mixed thoroughly and boiled before being dispensed into sterile bottles or maintained at about 50 °C before use in pour plates. Medium to be melted before use was placed in boiling water for 30 minutes then allowed to cool to 50 °C before use. One ml of an appropriately diluted meat sample was used to inoculate pour plates which were incubated at 30 °C.

Vacuum packed beef shin was stored at 5 °C for up to six weeks and sampled every two weeks. To minimise contamination the surface of a pack was wiped with 70% (v/v) ethanol prior to opening. Ten g were removed asceptically and homogenised with 90 ml of Maximal Recovery Diluent (MRD, Lab M) in a Colworth Stomacher for 60 s. A decimal

Letters in Applied Microbiology 1994, **18**, 327–328

A modification of the *Pseudomonas* selective medium, CFC, that allows differentiation between meat pseudomonads and Enterobacteriaceae

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GWG/228: accepted 4 January 1994

L.H. STANBRIDGE AND R.G. BOARD. 1994. Certain members of the family Enterobacteriaceae developing on beef steaks packaged in modified atmospheres may grow on the medium CFC (Cephaloridine-Fucidin-Cetrimide), which is selective for pseudomonads. The addition of arginine (1% w/v) and the pH indicator phenol red (0·002% w/v) to this medium differentiated between the two groups. The pseudomonads produced ammonia from arginine, whereas Enterobacteriaceae generally did not use this substrate. The alkaline drift in pH in and around pseudomonad colonies gave a pink colouration with phenol red.

INTRODUCTION

Pseudomonas spp. are important in the spoilage of chilled meats stored aerobically (Molin and Ternström 1982, 1986; Shaw and Latty 1982, 1984; Banks and Board 1983). In general, the CFC (cephaloridine-fucidin-cetrimide) medium of Mead and Adams (1977) permits the enumeration of such organisms even when they do not dominate a microbial association. During the course of an extensive study of meat packed under vacuum or in modified atmospheres, it was noted that members of Enterobacteriaceae grew on CFC, and gave inflated counts. The medium was amended by the addition of arginine and phenol red in order to overcome this problem. The present paper gives details about this modification.

MATERIALS AND METHODS

Modified CFC medium

CFC was made according to manufacturer's instructions but with the addition of 1% (w/v) L-arginine hydrochloride (Sigma) and 0·002% (w/v) Phenol Red (BDH) before autoclaving. The antibiotic supplement was added immediately prior to pouring into Petri dishes.

Enumeration of *Pseudomonas* spp. and Enterobacteriaceae

Vacuum-packed beef shin was stored at 5°C for up to 6 weeks and sampled every 2 weeks. The surface of a pack was wiped with 70% (v/v) ethanol to ensure an uncontaminated surface prior to opening the pack. Ten g were removed aseptically and homogenized with 90 ml of Maximal Recovery Diluent (MRD, Lab M) in a Colworth Stomacher for 60 s. A decimal dilution series using 9 ml amounts of MRD was made and 100 μl of the appropriate dilutions were spread onto CFC (Lab M) and modified CFC (incubation at 25°C for 48 h). With the latter, pink colonies were scored as *Pseudomonas* spp. and yellow ones were assumed to be Enterobacteriaceae. One ml of appropriate dilutions was used to inoculate pour plates of Violet Red Bile Glucose agar (Lab M) for enumeration of Enterobacteriaceae (incubation at 30°C for 48 h).

Isolation and characterization of isolates

Isolates (both pink and yellow) selected from modified CFC medium were streaked twice onto Nutrient Agar (NA, Lab M) to achieve purity. These were maintained on NA slopes in Bijou bottles at 5°C. Each isolate was tested for oxidase reaction (Kovac's reagent), Gram reaction, cell morphology and production of acid from glucose (modified Hugh and Leifson, as described in Harrigan and McCance, 1976). Isolates which were oxidase-positive, Gram-negative rods and produced acid from glucose oxidatively were deemed to be *Pseudomonas* spp. and oxidase-negative, Gram-negative rods that fermented glucose were presumed to be Enterobacteriaceae.

RESULTS AND DISCUSSION

The counts of all bacteria from samples of vacuum-packed beef throughout storage on CFC and modified CFC are shown in Fig. 1. The correlation (coefficient = 0·987) between the enumeration of bacteria from the two media

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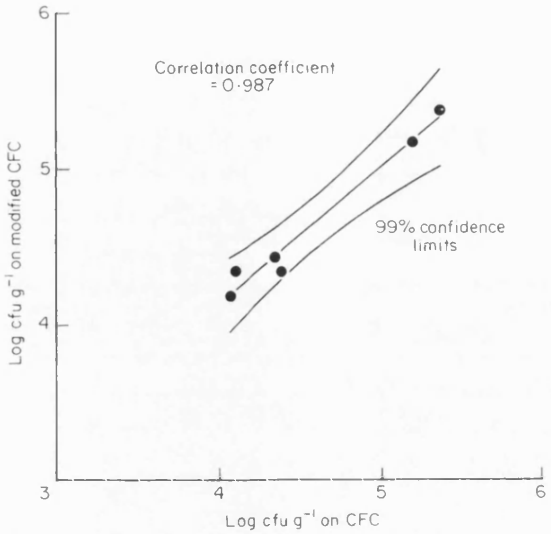

Fig. 1 Comparison of total bacterial numbers from pseudomonad selective media

shows that the addition of arginine did not affect the selectivity. At the outset, the beef samples contained relatively few Enterobacteriaceae (as determined by counts on VRBG) compared to pseudomonads (log 2·30 *vs* log 5·38, respectively). After 6 weeks colour differences in colonies growing on modified CFC were observed. In this case, the numbers of Enterobacteriaceae (VRBG counts) and pseudomonads (CFC counts) were equivalent (log 5·17 *vs* log 5·20, respectively). About equal numbers of colonies were present on CFC and modified CFC at this time (Table 1). A significant number of the colonies (yellow)

Table 1 Numbers of colonies present on cephaloridine-fucidin-cetrimide (CFC) and modified CFC from modified atmosphere-packaged beef stored at 5°C for 6 weeks

	Numbers of colonies		
Medium	Total	Pink	Yellow
CFC*	315	—	—
Modified CFC	301	184	127

* Numbers were taken from duplicate plates of one dilution.
—, Not applicable.

present on modified CFC were presumed to be Enterobacteriaceae. Isolates from a number of both colony types were characterized (Table 2). Only one of the 24 pink colonies was not a pseudomonad. In contrast, all 19 of the yellow ones were oxidase-negative and fermented glucose and were presumed to be Enterobacteriaceae.

The use of modified CFC allowed differentiation of pseudomonads and Enterobacteriaceae developing on meat stored in modified atmospheres for long periods. Problems occurred, however, with acid diffusion when modified CFC was inoculated with a spiral plating machine and with high inoculum levels. Acid produced by the Enterobacteriaceae caused yellowing of the medium and neutralized the alkaline drift of pseudomonads. Provided care was taken with the use of this modified medium, the amendment of CFC improved the accuracy of *Pseudomonas* counts in situations where Enterobacteriaceae numbers were liable to be high.

ACKNOWLEDGEMENT

This work was done as part of EEC Food-Linked Agro-Industrial Research project No. 89055.

Table 2 Characteristics of isolates from modified cephaloridine-fucidin-cetrimide

	Percentage of colonies showing these characteristics:			
Colony type	Oxidative	Fermentative	Oxidase +	Oxidase −
Pink ($n = 24$)	95·8	4·2	95·8	4·2
Yellow ($n = 19$)	0	100	0	100

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dilution series using 9 ml amounts of MRD was made and one ml was used to inoculate pour plates of VRBG and modified VRBG. Plates were incubated at 30 °C for 24 h.

RESULTS AND DISCUSSION

The numbers of bacteria recovered on modified VRBG were smaller than those on VRBG (Figure 2.8). As the addition of arginine was not expected to be inimical to organisms, the smaller number in modified media was unexpected. The low level of glucose present in the medium may have caused the apparent inhibition. There were no differences in colour (Figure 2.9) in the medium surrounding the colonies irrespective of their size. The modified VRBG was thus not effective as a selective differential medium.

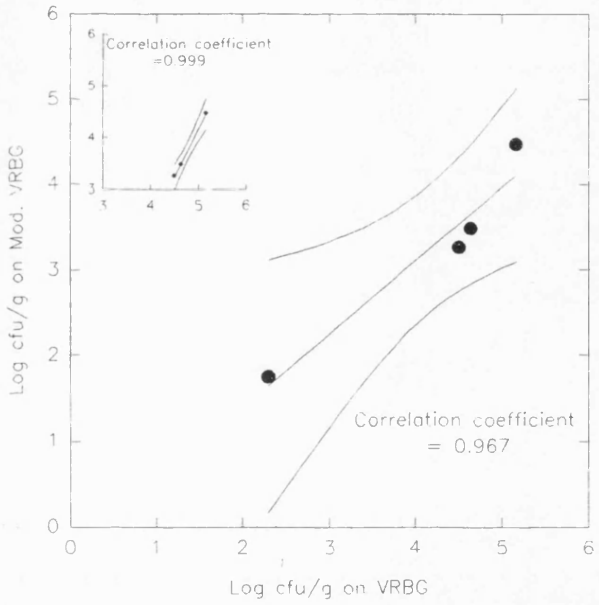


Figure 2.9 **Recovery of bacteria on VRBG and modified VRBG. Inset omits the lowest values. Confidence limits are 95%.**

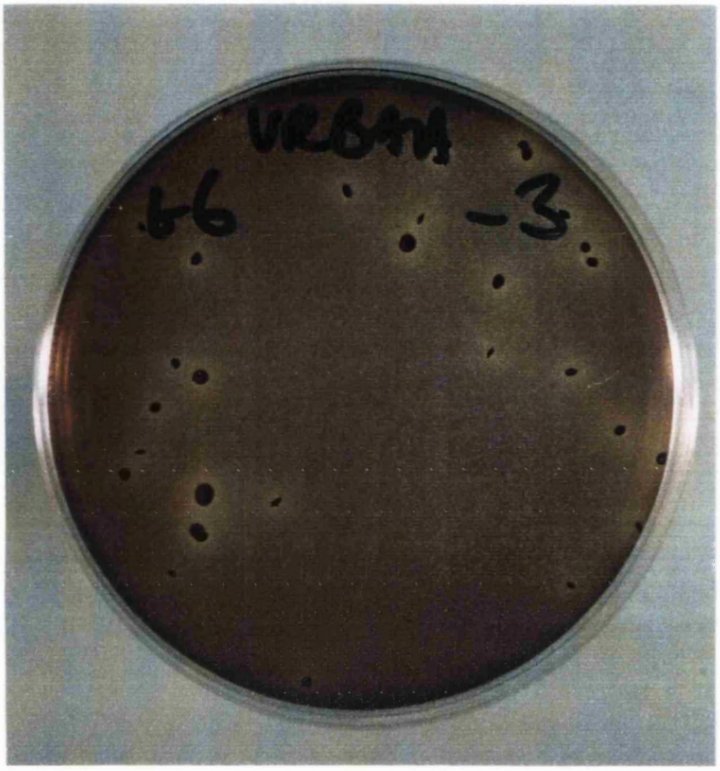


Figure 2.10 **Colony morphology on modified VRBG**
Colony size varied but no differences in colour were discernible

Pseudomonas selective-differential medium (BCP and BTB)

INTRODUCTION

The development of a selective differential medium to enumerate and presumptively identify the three species of meat *Pseudomonas* would enable rapid analyses of the structure of the pseudomonad community on MAP meat. Such a medium was devised. It was intended to identify the three species, *Pseudomonas fluorescens, Ps. lundensis* and *Ps. fragi.* The diagnostic attributes of the medium were based on production of fluorescence and acid from maltose. According to the taxonomic study by Molin and Ternström (1986), *Ps. fluorescens* and *Ps. lundensis* should fluoresce and the latter together with *Ps. fragi* produce acid from maltose. On an appropriate medium, acid-producing fluorescent organisms would identify with *Ps. lundensis*, those which fluoresced but did not produce acid with *Ps. fluorescens* and those which produced acid but did not fluoresce with *Ps. fragi.*

The chronology in the development of the selective differential medium is outlined below. Initially the important ingredients of CFC (the selective medium) were combined with the medium used for testing the production of acid from maltose (Hugh and Leifson, as described in Harrigan and McCance, 1976). As the extent of fluorescence on CFC was very low, optimisation of this property was essential. Many factors influence the production of pyoverdine, including the nature and concentration of the carbon and nitrogen sources, phosphate levels, oxygen concentration and the type and amount of trace elements present in the medium (Meyer *et al.*, 1987). The type of peptone used was found to have a significant effect on the production of pyoverdine also (King *et al.*, 1954). It is known that iron deprivation enhances fluorescence (Paton, 1959). Egg albumen which contains the iron-chelator, ovotransferrin, has been used for this purpose (Garibaldi, 1967). One ml of albumen binds approximately 21 µg iron.

EXPERIMENT 1

Materials and methods (All percentages are w/v unless otherwise stated)

The CFC medium (Lab M) was made according to the manufacturer's instructions and 0.006% bromocresol purple - BCP (BDH) and 1% maltose (BDH) added prior to autoclaving. The pH was about 7.1. The viscosity of egg white from recently laid eggs was

broken down in a Colworth Stomacher (Seward) for three minutes - to prevent foaming a minimum amount of air was present in the bag. Ten % (v/v) egg white was added to the medium immediately before pouring into Petri dishes. Strains of the three pseudomonad species (*Ps. fluorescens* biotype A LMG 1794, *Ps. fluorescens* biotype C LMG 5822, *Ps. fragi* LMG 2191 and a strain of *Ps. lundensis* isolated from meat) were inoculated onto the agar with a loop. The plates were incubated at 25 °C for 48 h.

Results and discussion

None of the pseudomonad strains produced acid from maltose. The glucose in the egg albumen (*ca* 0.4%) may have been the reason for the organisms failure to use the disaccharide. Fluorescence was observed with the strains of *Ps. fluorescens*, production being much greater in biotype A than C.

EXPERIMENT 2

Materials and methods

The above medium was used without the egg albumen. Inoculation and incubation conditions were as before.

Results and discussion

Again no acid production from maltose was observed. It was surmised that the buffering capacity of the medium together with alkaline products of peptone breakdown may have masked acid even if it had been produced.

EXPERIMENT 3

Materials and methods

The CFC medium and the medium used for testing the oxidative metabolism of maltose (Hugh and Leifson medium as described in Harrigan and McCance, 1976) were combined *viz.*: peptone 0.2, NaCl 0.5, K_2SO_4 1.0, $MgCl_2$ 0.14, BCP 0.006, maltose 1.0, and agar 1.5. CFC supplement was added before pouring the medium into Petri dishes. Inoculation and incubation of the plates was done as described previously. A Hugh and Leifson medium with 1.0% glucose rather than maltose was used as a control.

Results and discussion

Acid production caused a colour change (purple to yellow) around colonies of pseudomonads in the Hugh and Leifson medium containing glucose. Analogous acid production was observed in the modified medium. No fluorescence was observed. As noted previously the extent of fluorescence has been attributed in part to the degree of iron deprivation (Paton, 1959). It was possible that an excess of iron in the medium, either from the peptone or agar, was inhibiting fluorescence production in this medium.

EXPERIMENT 4

Materials and methods

Gelatin peptone is normally used in the CFC medium. It contains 4 ppm of iron *cf* the 88 ppm present in bacteriological peptone (David Post, pers. comm.). The above medium was prepared in order to compare normal bacteriological peptone with gelatin peptone (kindly provided by David Post, Unipath). Tissue culture agar (as used routinely) or the more highly purified Agar No. 1 (Oxoid) were also used. The effect of albumen (10% v/v) addition was examined also. After inoculation media were incubated at 25 °C for 48 h.

Results and discussion

The results of the amendments noted above (Table 2.5) revealed that all four strains of pseudomonads grew on media without egg white, only *Ps. fluorescens* biotype A grew on those containing albumen. When growth occurred acid was produced by *Ps. fragi* and *Ps. lundensis*. Fluorescence production occurred with *Ps. fluorescens* biotype A only. Even this strain did not produce the pigment in the medium containing the gelatin peptone, agar no. 1 and albumen. It is probable that this organism was able to fluoresce with the low iron levels present in the media containing albumen.

Table 2.5 Reactions of pseudomonads strains to variations of BCP medium

Medium	Growth[a]	Acid from Maltose	Fluorescence
Peptone + Tissue Culture (TC) Agar	ALL	1,2	NONE
Peptone + TC Agar + Albumen	4	NONE	4
Peptone + Agar No.1	ALL	1,2	NONE
Peptone + Agar No.1 + Albumen	4	NONE	4
Gelatin Peptone + TC Agar	ALL	1,2	NONE
Gelatin Peptone + TC Agar + Albumen	4	NONE	4
Gelatin Peptone + Agar No.1	ALL	1,2	NONE
Gelatin Peptone + Agar No.1 + Albumen	4	NONE	NONE

a 1, *Ps. fragi;* 2, *Ps. lundensis*; 3, *Ps. fluorescens* biotype C; 4, *Ps. fluorescens* biotype A

EXPERIMENT 5

Materials and methods

As no differences were noted with the type of peptone or agar, the medium using bacteriological peptone and tissue culture agar (described above) was used. A range of albumen concentrations (0, 0.1%, 0.25%, 0.5%, 0.75%, 1.0%, 2.0%, 5.0%, 7.5% and 10% v/v) was included in the medium.

Results and discussion

Albumen concentrations of between 0 and 2% gave positive reactions for acid from maltose, whilst levels 5% and above did not. Fluorescence was observed at albumen levels between 2 and 10%. The two percent albumen concentration therefore gave both acid reactions and fluorescence on the same medium (Figure 2.10) but neither reaction was particularly strong.

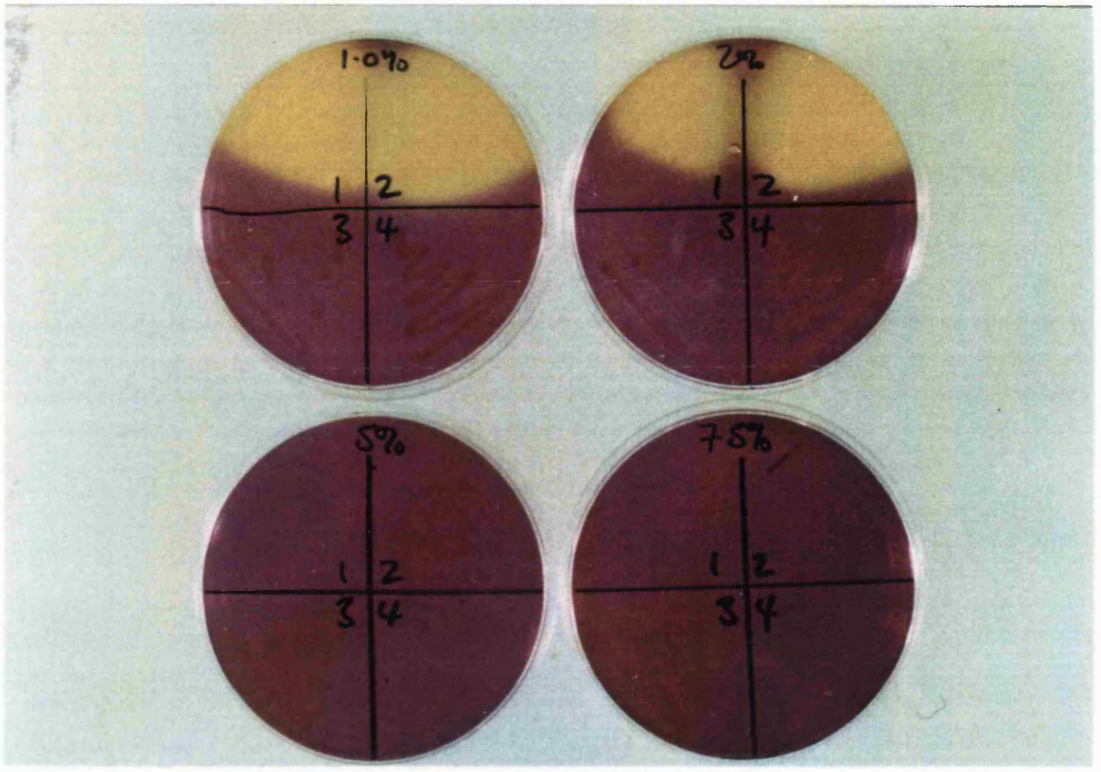
EXPERIMENT 6

Materials and methods

The variation in albumen concentrations was repeated with a narrower range (2-5% at 0.5% [v/v] intervals). Variations in other medium constituents were also tested (Table 2.5).

Results and discussion

The results showed that an albumen concentration of 2.0% gave acceptable reactions for acid from maltose and a weak positive reaction for fluorescence (Table 2.6). The lowest level of gelatin peptone was associated with most marked acid reaction; very low levels of fluorescence were observed with the higher levels of the peptone (Table 2.6). The omission of salt from the medium appeared to improve both the extent of acid production and fluorescence.



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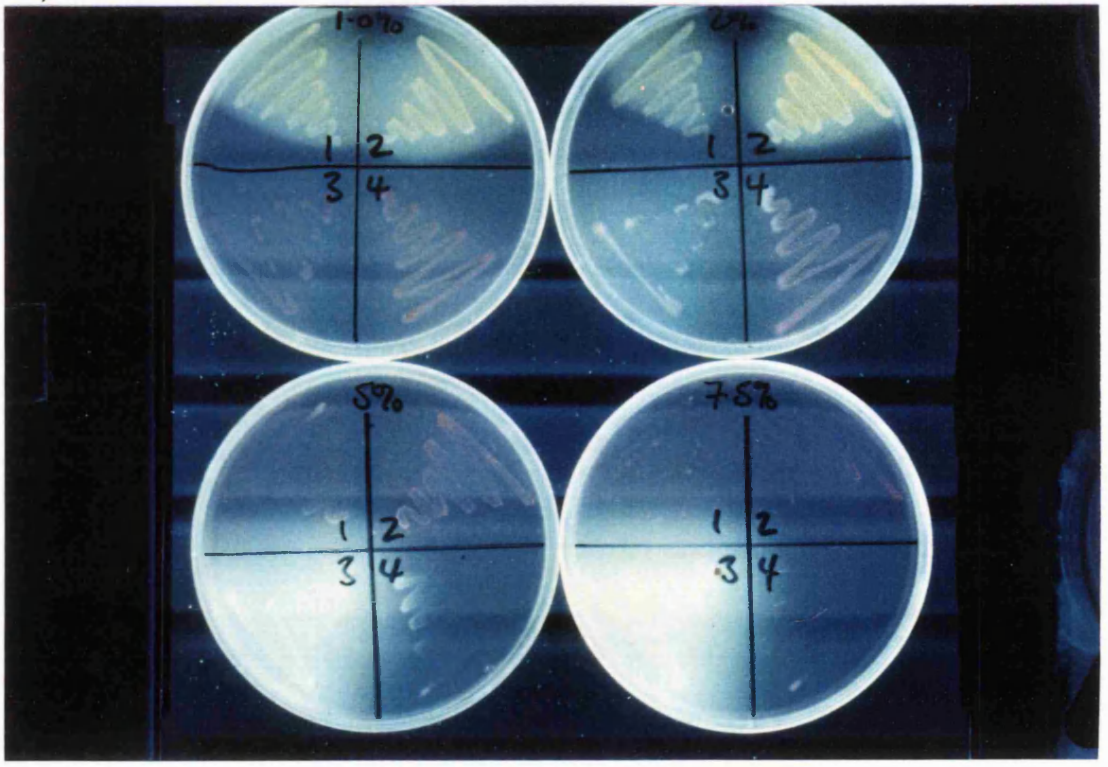


b)

Figure 2.10 **Characteristics of the *Pseudomonas* selective-differential medium, BCP containing different concentrations of egg white (1.0, 2.0, 5.0 and 7.5%)**

a) Production of acid from maltose

b) Fluorescence under UV

1 *Ps. fragi*, 2 *Ps. lundensis*, 3 *Ps. fluorescens* biotype A, 4 *Ps.fluorescens* biotype C

Table 2.6 Variations in media components in BCP*.

Media component	(% w/v)	Acid from Maltose	Fluorescence
Albumen	2.0	+++	+
	2.5	++	+
	3.0	++	+
	3.5	++	+
	4.0	++	+
	4.5	+	+
	5.0	-	+
Gelatin Peptone	0.2	++	+/-[a]
	0.9	-	++
	1.6	-	+++
BCP	0.003	++	++
NaCl	0	++	++
Maltose	0.5	-	++
Peptone	1	-	+++

a very faint positive reaction

* All other components of the normal concentration (Experiment 3, pp. 54)

EXPERIMENT 7

Materials and methods

In all experiments to date, strains previously grown in nutrient-rich media were heavily inoculated with a loop onto a medium. With environmental samples the reactions of isolates forming single colonies may differ from those of laboratory strains because of size of biomass at the time of inoculation and phenotypic changes due to extended storage. Thus, an experiment with CFC, BCP and bromothymol blue (BTB) to compare the reactions of isolates from minced beef was done. The BCP and BTB media were made as before but with 0.006% BTB and 2% albumen. Two samples of MAP minced beef were purchased from local shops. Ten g of mince were homogenised with 90 ml MRD (Lab M) for 60 s in a Colworth Stomacher. A decimal dilution series was made and 100 µl of appropriate dilutions were spread onto the surface of CFC, BCP, BTB and PCA (total viable count). Duplicate plates were inoculated with each dilution. All plates were incubated at 25 °C for up to 3 days. The BTB plates were monitored daily.

Results and discussion

This experiment showed that comparable counts were obtained with all three selective media (Table 2.7). The largest proportion of fluorescent colonies was detected with the CFC medium rather than with either of the selective differential media. Of the last two, the most rapid colour changes from acid production occurred on BTB and these were more easily seen than those occurring on BCP. Acid diffusion away from a colony was a

problem because it could give false-positive results - colonies of non-acid producers were surrounded by media acidified by other colonies.

Table 2.7 Comparison of three media for differentiation of *Pseudomonas* species

Medium	Meat sample	Bacterial numbers (log cfu/g) Total	Fluorescent	Acid	Non-acid
CFC	A	4.65	4.19	-[a]	-
	C	4.38	3.30	-	-
BCP	A	4.43	3.15	2.95	4.24
	C	4.20	2.36	3.90	3.77
BTB	A	4.30	2.98	3.90	3.96
	C	4.15	2.98	4.08	3.48

a Acid can not be detected on CFC medium

EXPERIMENT 8

Materials and methods

A range of organisms (Table 2.7) from the culture collection maintained at the University of Bath were used to check the selectivity of CFC and BTB. The micro-organisms were streaked onto each medium. All media were incubated at 25 °C and results recorded after 48 h.

Results and discussion

Table 2.8 lists the organisms which grew on CFC and/or BTB. *Hafnia alvei, Serratia liquefaciens, Ser. marcescens, Proteus vulgaris* and *Aeromonas hydrophila* grew on both CFC and BTB. *Shewanella putrefaciens* grew on CFC but not on BTB. All organisms capable of growth on BTB also produced acid on this medium. In some circumstances therefore there could be inflated counts in studies of pseudomonads on meat. As both organisms are common contaminants on meats, it is of interest to note that *Haf. alvei* and *Ser. liquefaciens* grew on CFC.

EXPERIMENT 9

The use of BTB in identification of pseudomonad species from meat, poultry and fish samples.

Table 2.8 Selectivity of pseudomonad media

Organism	Medium of growth: PCA	CFC	BTB
Acinetobacter sp.	+[a]	-	-
Aeromonas hydrophila	+	+	+
Bacillus cereus	+	-	-
subtilis	+	-	-
Brochothrix thermosphacta	+	-	-
Enterobacter aerogenes	+	-	-
Enterococcus faecalis	+	-	-
faecium	+	-	-
Escherichia coli	+	-	-
Hafnia alvei	+	+	+
Kurthia zopfii	+	-	-
Lactococcus lactis	+	-	-
Pantoea agglomerans	+	-	-
Proteus vulgaris	+	+	+
Salmonella waycross	+	-	-
Serratia liquefaciens	+	+	+
marcescens	+	+	+
Shewanella putrefaciens	+	+	-
Staphylococcus aureus	+	-	-

a +, growth; -, no growth

Materials and methods

The BTB medium had the following composition: bacteriological peptone (0.2%), K_2SO_4 (1.0%), $MgCl_2$ (0.14%), BTB (0.006%), maltose (1.0%) and agar -tissue culture (1.5%). Microbiological enumeration of food samples was done by cutting them into small pieces and homogenising 10 g with 90 ml of Maximal Recovery Diluent (MRD, Lab M) for 60 s in a Colworth Stomacher (Seward). A decimal dilution series was made in MRD (1 ml of the homogenate + 9 ml MRD) and appropriate dilutions were spread (0.1 ml) onto the surface of BTB and CFC.

Results and discussion

The regression analysis of numbers from BTB and from CFC (Figure 2.11) shows comparable recovery of pseudomonads on the two media. Hence, BTB could be used as an alternative to CFC in the enumeration of pseudomonads in proteinaceous food samples.

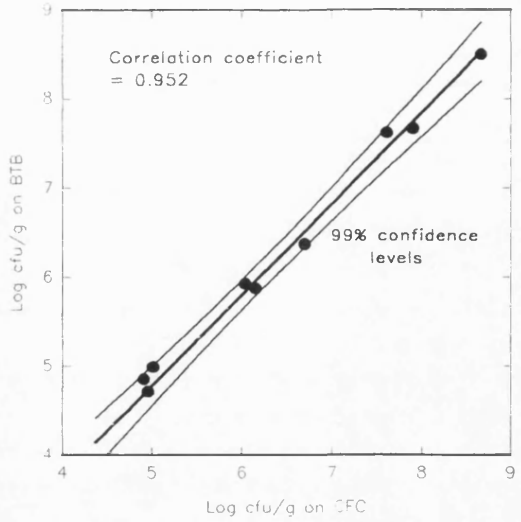


Figure 2.11 The recovery of pseudomonads from two meat selective media

EXPERIMENT 10

BTB and the presumptive identification of *Pseudomonas* species on meat samples.

Materials and methods

Minced beef from three local butchers were sampled as described above and appropriate dilutions spread onto CFC and BTB. Bacteria were isolated from the media, a sterile toothpick being used to transfer colonies onto Nutrient agar (Lab M). An isolate was streaked twice to ensure purity, then identified using the protocol given on pages 149-151. Ninety isolates (30 from each sample) were purified from each medium. The colonies on BTB were described at the time of isolation.

Results and discussion

The weighted means for counts on the two media are given in Table 2.9. As noted previously, there was very little difference in the counts obtained.

Table 2.9 Numbers of bacteria isolated from minced beef samples on selective media

Medium	Weighted mean of bacterial recovery from samples (log cfu/g):		
	A*	B	C
CFC	6.99	7.86	4.99
BTB	6.98	7.91	4.99

* A, B and C were meat samples bought from three local butchers

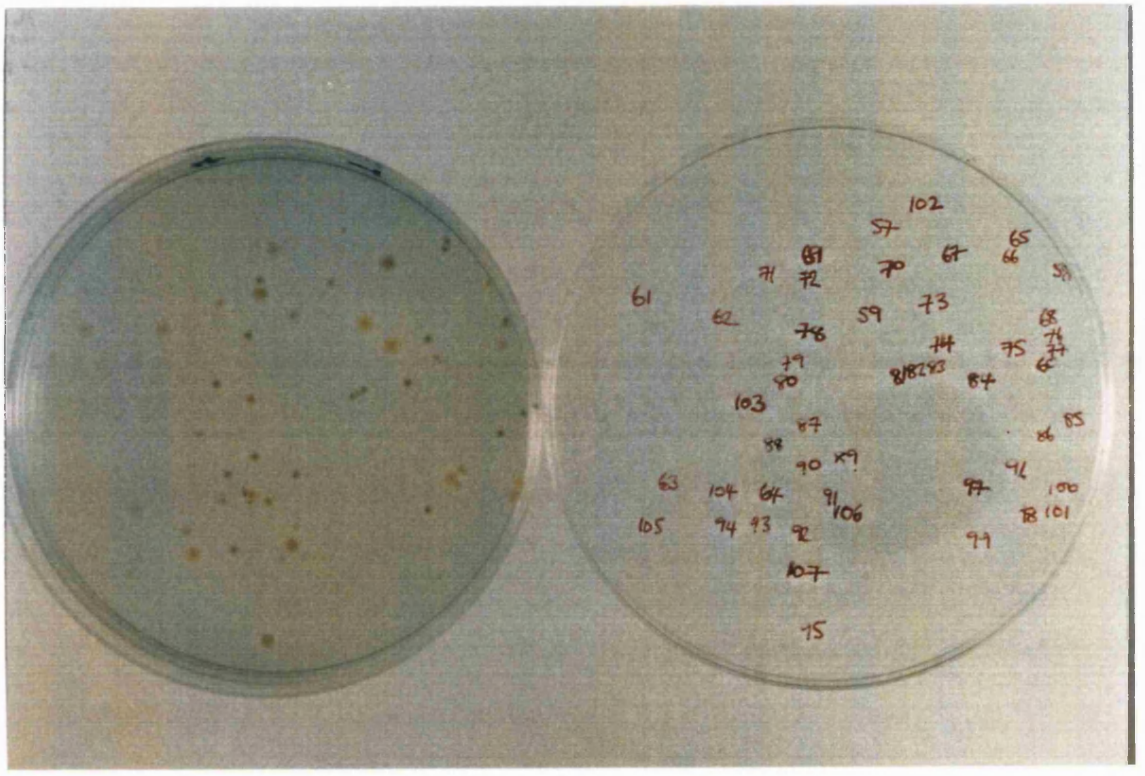
The colony types were difficult to characterise as fluorescence levels were low (Figure 2.12). The results (Table 2.10) showed that prediction of species identified from colony form on BTB did not correlate with the identification from subsequent studies. In some cases, colonies predicted as *Ps. lundensis* were *Ps. fragi*, indicating that the test for fluorescence was unreliable. This was probably due to the refraction of light from colonies on media observed under UV.

Table 2.10 Analysis of *Pseudomonas* species from different selective media

	CFC			BTB			Predicted
	A*	B	C	A	B	C	accurately (BTB)
Ps. fragi	26	24	23	22	27	20	82.1%
Ps. fluorescens	1	1	4	6	2	7	33.3%
Ps. lundensis	2	1	0	2	1	2	0%
Unidentified	1	2	3	0	0	1	NA**

* A, B and C were meat samples bought from three local butchers

** NA Not applicable



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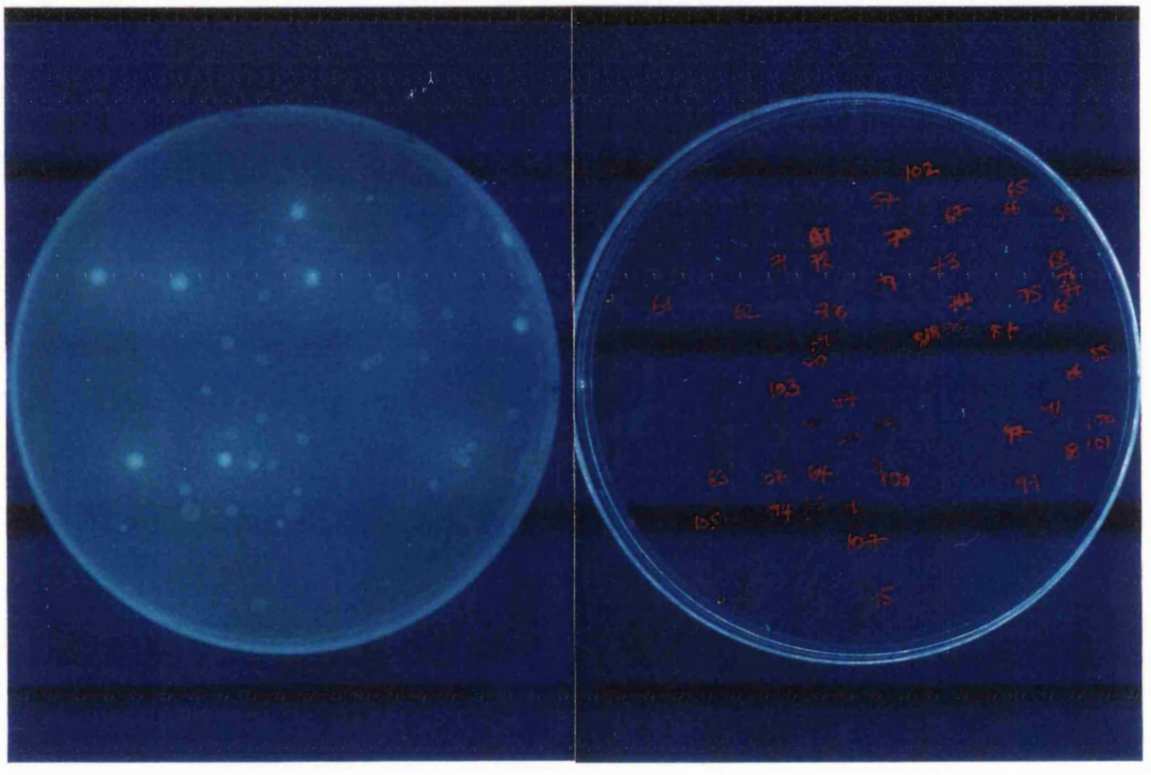


b)

Figure 2.12 ***Pseudomonas*** **selective-differential medium, BTB, in the identification of isolates from meat**

a) Production of acid from maltose

b) Fluorescence under UV

Colonies were isolated and identified according to the methods described (pp.149-151)

Discussion

The use of BTB medium did not allow an acceptable level of identification of the *Pseudomonas* spp. important in meats or in the environment of meat processing plants. The colony types were difficult to distinguish and fluorescence levels were relatively low. The production of the fluorescent pigment, pyoverdine, by *Ps. fluorescens* is known to be affected by many factors, e.g. the organic carbon and energy source, pH, light and cations (King *et al.*, 1954). The level of iron (Fe III) in a medium is crucial (pp. 48). This is connected with its function as a siderophore - a "low molecular weight (500-1000 D) virtually ferric specific ligand, the biosynthesis of which is carefully regulated by iron and the function of which is to supply iron to the cell" (Neilands, 1981). Meyer and Abdallah (1978) found that the small amount of contaminating iron in a minimal medium inhibited excretion of pyoverdine in the stationary phase of the growth cycle. The metal was, however, found to be necessary for pyoverdine production by *Ps. aeruginosa* (Barbhaiya and Rao, 1985). Garibaldi (1967) suggested 10% egg white be added to media for the enhancement of fluorescence as the iron is chelated by the protein ovotransferrin. At this concentration the production of acid from maltose was prevented in the present study.

The level of oxygen in the medium was found to affect pyoverdine production, with media which were shaken and contained low iron concentrations giving higher pigment levels than media in other conditions (Lenhoff, 1963). The volume of static liquid media compared to size of flask was found to be an influencing factor (Barbhaiya and Rao, 1985). The atmosphere of incubation was not tested in the present study.

The pigment is stable at pH 7.3, but breaks down in mildly alkaline conditions. This should not have been a problem with the BTB medium at pH 7.1. The concentration of trace metal ions are important in the optimisation of pyoverdine production. Magnesium was found to be essential whilst zinc, copper, nickel and manganese stimulated pigment production at low concentrations (Chakrabarty and Roy, 1964). The media constituents used in the BCP and BTB media were not analysed to determine the concentrations of metal ions. In the study by Barbhaiya and Rao (1985), a variety of carbon sources were tested to determine their effect on growth and pyoverdine concentration. No pigment was detected when maltose was the substrate. Their study was done with *Ps. aeruginosa*, and the results may differ with *Ps. fluorescens* and *Ps. lundensis*. The present study was able to detect both pyoverdine and production of acid from maltose, unfortunately the results did not allow suitable identification of the meat pseudomonads.

CHAPTER 3

PACKAGING EXPERIMENTS - AN OVERVIEW

INTRODUCTION

As described in the Literature Review (pp. 2-32), meat packaged in modified atmospheres (MAP) has a longer shelf life than that overwrapped with protective films (e.g. Gill and Molin, 1991; Hood and Mead, 1993). There is uncertainty, however, about the safety of MAP meat. As the aerobic spoilage bacteria, the major cause of offensive off-odours of meat stored in air are inhibited, food-borne pathogens may increase in numbers before the odours produced by the facultatively anaerobic organisms are manifest (Hintlian and Hotchkiss, 1987). In other words MAP does not exhibit a fail-safe end point. This concern was central to the EEC Food-Linked Agro-Industrial Research programme entitled "Improving the safety and quality of meat and meat products by modified atmospheres and assessment by novel methods". Partners in the programme studied the behaviour of a range of food-borne pathogens (*Aeromonas hydrophila*, verotoxigenic *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Yersinia enterocolitica*) in lamb, beef or chicken stored at 0 or 5 °C in a range of modified atmospheres. Members of the microbial consortium developing on the meat during storage were enumerated (the media are listed in Table 3.1). Detailed characterisation of the microbial community in three trials (one with *Listeria monocytogenes* and two with *Salmonella typhimurium*) was done at Bath University. The results presented in this section exemplify those obtained in the course of the study. They provide the background against which the results of the microbial analysis of the populations can be viewed.

MATERIALS AND METHODS

Experimental design

Beef steaks, obtained from a local slaughterhouse after ageing, were dipped in cell suspensions of food-borne pathogens. After overnight storage at 0 °C steaks were packed in atmospheres of 80% O_2 + 20% CO_2, 50% N_2 + 50% CO_2, 100% CO_2 or in vacuum, stored at 0 or 5 °C and sampled at intervals up to 34 (5 °C) or 77 (0 °C) days. The day of packaging - 4 days *post mortem* - was taken as day 0.

Meat supply

Steaks were cut from topsides obtained from Fresian-Limousin cross beef cattle (10-11 months old) at an EC-approved abattoir. Over a two-day period the carcasses were monitored for temperature and pH levels. The carcasses were butchered on site and the steaks (250g; approximately 14 x 11 x 1.5 cm) transported in a refrigerated lorry to the laboratory and randomised upon receipt.

Inoculation

Listeria monocytogenes NCTC 11994 and *Salmonella typhimurium* were grown in tryptone soy broth or on agar (both Oxoid) at 30 °C. A cocktail of *S. typhimurium* strains 7M-4987, S-5698, 77-7628, 7M-5522 all resistant to 50 μg ml^{-1} nalidixic acid and 1000 μg ml^{-1} streptomycin (Blackburn and Davies, 1993) were used. All organisms were subcultured twice before use. The organisms were diluted in sterile distilled water to produce an inoculum of approximately 10^6 cells ml^{-1}. The steaks were dipped in the inoculum for 5 seconds using flame-sterilised forceps, then drained. The meat was stored overnight at 0 °C before packaging. Pathogens were enumerated using methods listed in Table 3.1.

Packaging

Steaks were vacuum-packed in Suprovac 90 vacuum pouches (90 μm thick, Kempner Ltd., Perry Road, Industrial Estate East, Witham, Essex. CM8 3TY). The gas permeability of the pouches was OTR *ca* 25, CDTR ca 90 and NTR ca 6 $m^3 m^{-2} day^{-1} bar^{-1}$ at 20 °C and 50% relative humidity (RH). The permeability of water vapour was *ca* 1.1 $g^{-2} d^{-1}$ at 23 °C and 85% RH. The settings for the Suprovac 180 vacuum packer (Berkel Ltd., 72, Cobden Street, Leicester. LE1 2LE) were: full vacuum, vacuum time setting of 8 and seal temperature setting of 9. Steaks, in high density polyethylene trays (Dynopack, Orion House, Calleva Industrial Park, Aldermaston, Reading, Berkshire. RG7 4QW) were packed in modified atmospheres using a Mecapac M500 machine (Swissvac, Unit A, Marish Wharf, St. Mary's Road, Langley, Berkshire. SL3 6DA) with a Kempner Suprovac 90 top web (permeability data as for vacuum pouches). The pre-mixed food-grade gases (80% O_2 + 20% CO_2, 50% N_2 + 50% CO_2 or 100% CO_2) were supplied by BOC (Unit 1C, Roxborough Way, Maidenhead, Berkshire. SL6 3UD). Approximately 2 litres of gas were added per kg of meat.

Storage

Packs were stored at 0 or 5 °C and removed for analysis at intervals up to 34 (5 °C) and 77 (0 °C) days.

Microbiological evaluation

The numbers of lactic acid bacteria and the presence/absence of the pathogens on the carcasses in the slaughterhouse were determined. A 20 g sample excised from the left-hand side of each carcass was added to 180 ml of maximum recovery diluent (MRD; Oxoid. CM733). The sample was stomached (Colworth Stomacher; Seward) for 60 seconds and a decimal dilution series prepared in MRD. Appropriate dilutions were inoculated onto selective media (Table 3.1). The inoculum of 0.5 ml was used to isolate the pathogens. In the case of *Listeria*, 25 g was removed from the carcass and examined for presence/absence by the revised USDA method (McClain and Lee, 1989).

The composition of the microflora on two steaks stored in each atmosphere was determined at every sampling time. A steak was quartered and two of the latter were cut into small pieces under aseptic conditions. Twenty g from each of the two quarters were added to 180 ml of MRD and a dilution series was prepared as described previously. After enumeration one of each duplicate set of plates was sent to Bath University for detailed characterisation of isolates.

Gas composition

This was measured with a Gow Mac Gas Chromatograph (Model 5292-202). Ten ml of gas were removed from the pack with a syringe needle passed through a silicone rubber seal. The percentage of each gas was calculated by extrapolation of data from gas standards. The percent nitrogen was calculated by difference.

Sensory evaluation

Two trained panellists assessed the organoleptic properties of the meat 30 min after the storage atmosphere had dissipated. One hour after opening, the meat on the polythene trays was wrapped with oxygen permeable cling film and stored for between

Table 3.1 Media used in microbiological testing of meat deliberately inoculated with a pathogen and in uninoculated controls

Medium[a]	Method[b]	Incubation conditions			Bacteria enumerated
		°C	(d)	atmosphere[c]	
APT	Spiral	25	3	Aer	Total aerobic
APT	Spiral	25	3	An	Lactic acid bacteria
CFC	Spiral	25	3	Aer	*Pseudomonas* spp.
STAA	Spiral	25	2	Aer	*Brochothrix thermosphacta*
VRBG	Pour (1ml)	30	2	Aer	Enterobacteriaceae
OXFORD	Spiral	30	2	Aer	*Listeria monocytogenes*
XL	Spiral	30	2	Aer	*Salmonella typhimurium*
TSA/TSB	Various	30	1/2	Aer	General culture medium

a APT All Purpose Tween (Difco); CFC Cephaloridine, Fucidin, Cetrimide (Lab M); STAA Streptomycin Thallous Acetate (Oxoid); VRBG Violet Red Bile Glucose (Oxoid); OXFORD Listeria selective agar, Oxford formulation (Oxoid); XL Xyline lysine agar (Difco) with addition of 50 µg/ml nalidixic acid and 1000 µg/ml streptomycin; TSA Tryptone Soy Agar/Broth - used for the general culturing of micro-organisms.

b Spiral plater (Don Whitley Scientific, 14, Otley Road, Shipley, West Yorkshire. BD17 7SE) used according to the manufacturer's instructions

c Aer, aerobic; An, anaerobic (Oxoid gas generating kit)

two and five days at the same temperature as that at which the steaks had been stored initially.

Odour: The odour (e.g. beef, sweet, cheesy) of raw meat was described. The product was considered spoiled when the odour was no longer considered to be typical of that of fresh beef. A modified boiling test was used to assess the odour of the cooked meat. About five g of a chopped sample were placed in a beaker with 15 g of distilled water. The beaker was covered and put over a Bunsen flame until the water boiled for 3 - 5 minutes. The cover was removed and two trained panellists described the odour as well as the appearance of the meat. The meat was considered spoiled when the odour was no longer like that of cooked beef.

Colour/Appearance: The colour of the surface of the meat was described as dark red, red, pale red etc. and any unusual features noted also. When the surface was heterogeneous in colour, the approximate percentage of each colour was noted.

RESULTS

Gas composition of the packs changed during storage (Table 3.2). Deliberate inoculation of meat with a pathogen did not have a demonstrable effect on the numbers of non-pathogenic bacteria or on the relative proportions of major taxonomic groups in the microbial associations. In other words, the results for inoculated steaks were similar to those for uninoculated ones. Gill and DeLacy (1991) also found a common microflora on control steaks and those inoculated with pathogens. The results discussed below are for uninoculated steaks.

Table 3.2 Changes in the composition of the atmosphere in MAP beef steaks during storage at 0 or 5 °C

Initial gas composition	Gas composition at the end of storage (approximate %)					
	0 °C			5 °C		
	O_2	CO_2	N_2	O_2	CO_2	N_2
50% N_2 + 50% CO_2		35	65		42	58
80% O_2 + 20% CO_2	50	45	5	40	57	3
100% CO_2		80	20		83	17

The microbial flora developing on uninoculated beef steaks at 0 and 5 °C are presented in Figures 3.1 and 3.2 respectively. The spoilage flora was similar at both temperatures. It was dominated by lactic acid bacteria (LAB). *Brochothrix thermosphacta* attained numbers equivalent to those of the LAB in the 80% O_2 + 20% CO_2 atmosphere, but lower levels in the CO_2 enriched atmospheres (50%N_2 + 50% CO_2 and 100% CO_2). Its growth was inhibited in VP. The numbers of pseudomonads and Enterobacteriaceae did not change or decreased at 0 °C. At 5 °C their numbers increased slightly, except in the 100% CO_2 atmosphere. In the high oxygen atmosphere, the populations of both reached approximately 10^7 cfu g^{-1}. Similar trends were noted in repeat experiments, although the level of growth of groups other than the LAB did vary on occasions. For example, in a trial at 5 °C, the extent of growth of pseudomonads and Enterobacteriaceae was greater than those described above, even though the initial level of overall bacterial contamination was more than 1 log lower.

The sensory attributes of the meat used in the initial trial (Tables 3.3 and 3.4) showed that the VP gave the longest shelf-life - >70 days - at 0 °C. This form of packaging is unsuitable for retail use because of the development of colour unacceptable to the consumer and the "squashed" appearance of the meat. Meat in all modified

atmospheres with storage at 0 °C were unacceptable after 56 days. At 5 °C 100% CO_2 - the most inhibitory atmosphere to the spoilage flora - the shelf-life was extended to >34 days as it was also in VP at this temperature. The packs containing carbon dioxide alone collapsed. The first signs of this state were apparent after 2 days at 5 °C and by the 8th day the top web was in contact with the meat surface. Early stages in pack collapse were also observed with the other atmospheres, for example after 10 days in 50% N_2 + 50% CO_2 and 20 days in 80% O_2 + 20% CO_2 with storage at 5 °C.

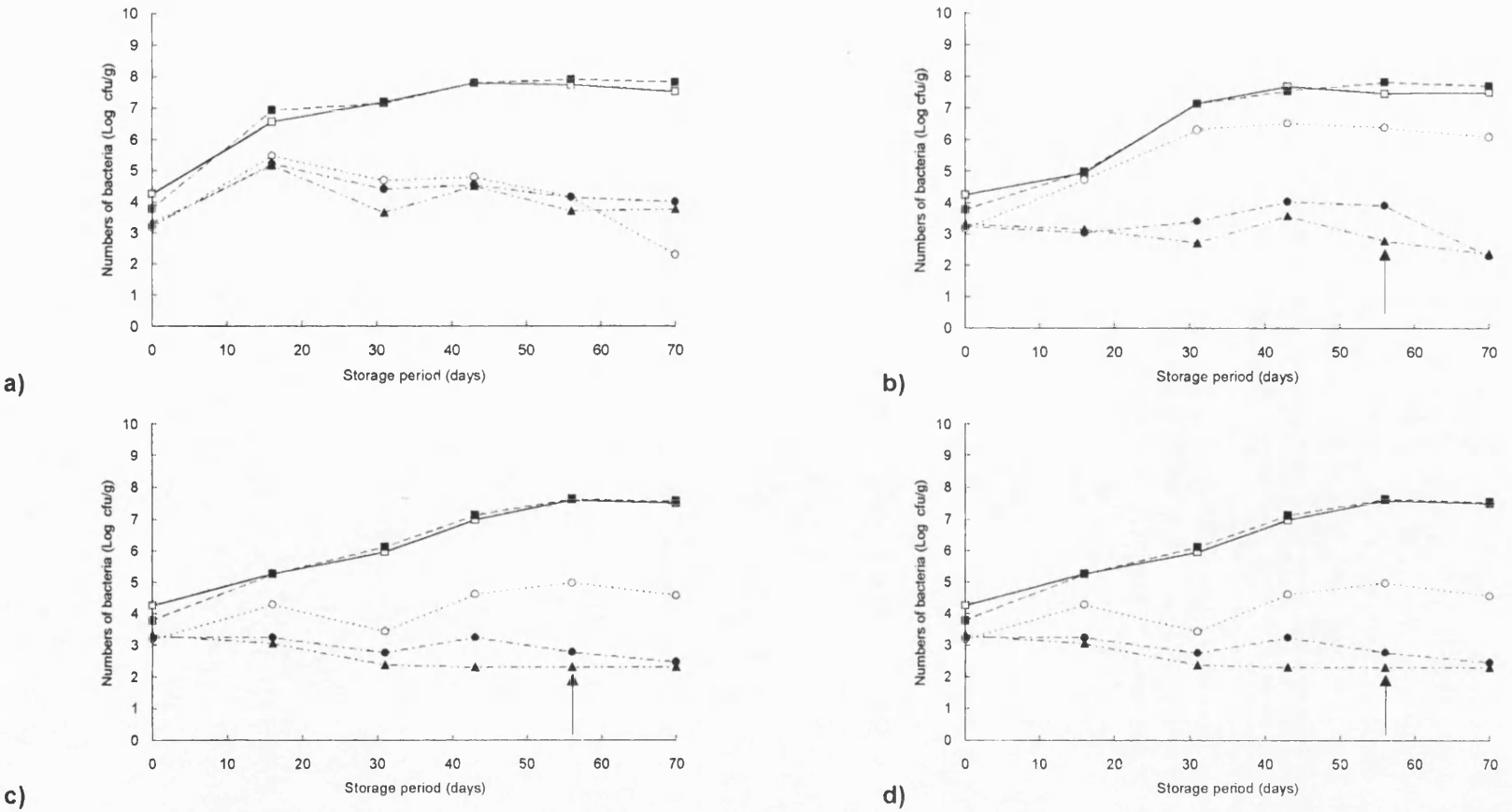


Figure 3.1 **Microbial flora developing on beef steaks stored at 0 °C in; a) vacuum pack; b) 50% N_2 + 50% CO_2; c) 80% O_2 + 20% CO_2, or d) 100% CO_2**

□ Total aerobic count; ■ Total anaerobic count; O *Brochothrix thermosphacta*; ● *Pseudomonas* spp.; ▲ Enterobacteriaceae
Arrow, the first sampling time at which the steaks were considered unacceptable by sensory evaluation.

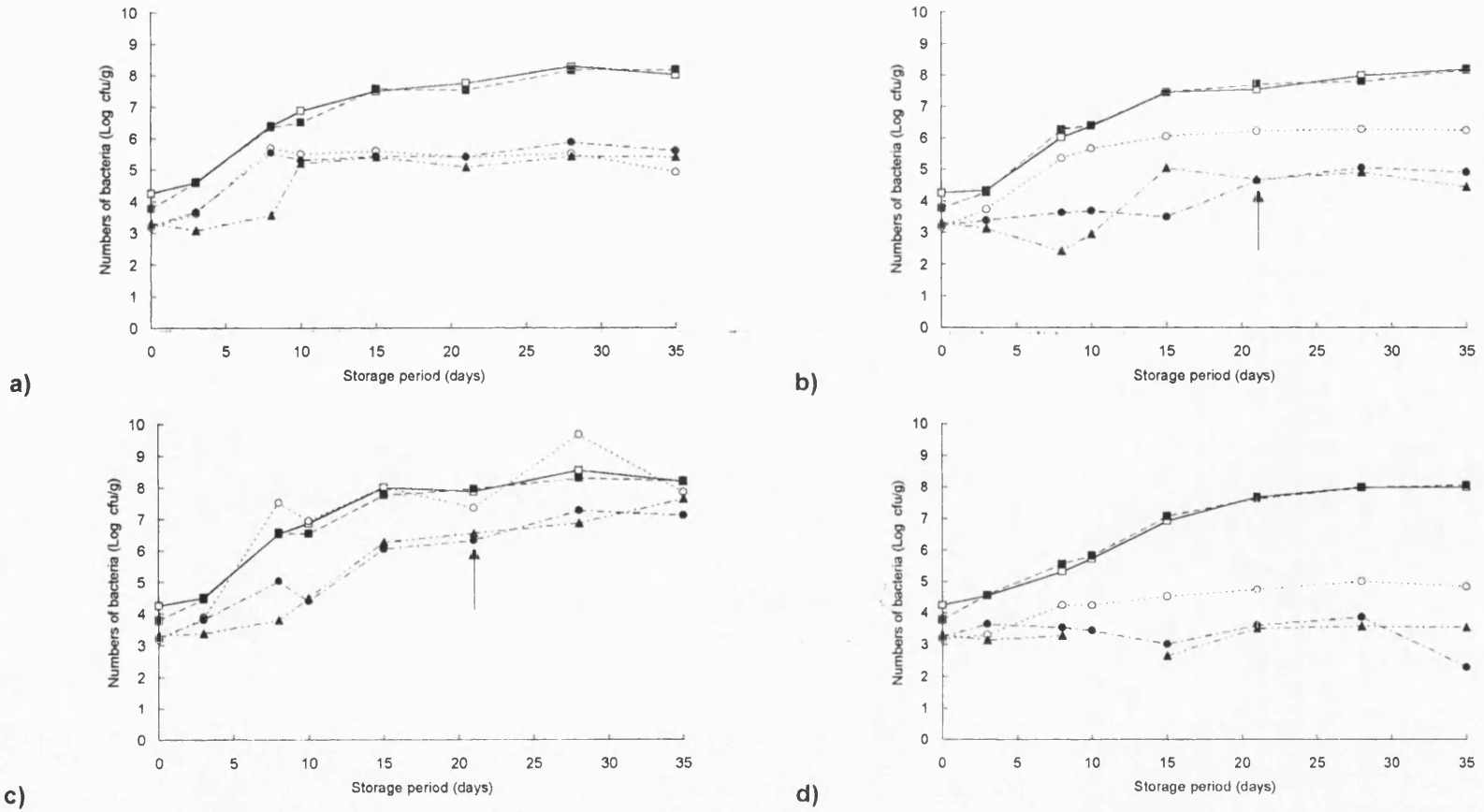


Figure 3.2 **Microbial flora developing on beef steaks stored at 5 °C in; a) vacuum pack; b) 50% N_2 + 50% CO_2; c) 80% O_2 + 20% CO_2 or d) 100% CO_2**

□ Total aerobic count; ■ Total anaerobic count; ○ *Brochothrix thermosphacta*; ● *Pseudomonas* spp.; ▲ Enterobacteriaceae

Arrow, the first sampling time at which the steaks were considered unacceptable by sensory evaluation.

Table 3.3 Spoilage characteristics of MAP beef steaks stored at 0 °C

		Modified atmosphere		
	VP	50% N_2 + 50% CO_2	80% O_2 + 20% CO_2	100% CO_2
Shelf-life[a]	>70	56	56	56
Odour	Beef	Slight acidic	Stale	Slightly sweet
Colour	Dark red	Pale brown	Pale brown	80% Red 20% Brown
Dominant organisms*	LAB	LAB	LAB/Br	LAB

a First day on which the sample was considered unacceptable by sensory evaluation.
* LAB Lactic acid bacteria Br *Brochothrix thermosphacta*

Table 3.4 Spoilage characteristics of MAP beef steaks stored at 5 °C

		Modified atmosphere		
	VP	50% N_2 + 50% CO_2	80% O_2 + 20% CO_2	100% CO_2
Shelf-life[a]	>34	21	21**	>34
Odour	Beef	Sweet	Stale	Beef
Colour	Red	50% Brown 50% Red	Pale brown	Red
Dominant organisms*	LAB	LAB	LAB/Br	LAB

a First day on which the sample was considered unacceptable by sensory evaluation.
* LAB Lactic acid bacteria Br *Brochothrix thermosphacta*
** On day 24 the odour was of beef and the steak was red in colour

An overview of effect of storage on modified atmosphere packaged meats on the growth of pathogenic organisms is given in Table 3.5. None of the atmospheres tested provided conditions suitable for the growth of the pathogens on beef at 0 and 5 °C. At the severe abuse temperature of 12 °C, *Escherichia coli* grew in the 80% O_2 + 20% CO_2 atmosphere and *Salmonella typhimurium* grew in the high oxygen atmosphere and in vacuum packs. The high oxygen atmosphere did, however, retard the growth of *Esch. coli* when compared to that in an aerobic environment on similar meat.

The pathogens tended to grow better in modified atmospheres on lamb (Table 3.5). Each of the pathogens tested (*Aeromonas caviae, Listeria monocytogenes, Yersinia enterocolitica*) grew in one or more of the atmospheres at 5 °C. The last named pathogen also grew at 0 °C in the vacuum pack and 50% N_2 + 50% CO_2 atmosphere. Growth of pathogens at 10 °C was noted also on MAP chicken. In this case, however, *Sal. enteritidis* grew in vacuum packs, the high oxygen atmosphere and in 100% N_2, an atmosphere used specifically for chicken in this study. Its growth was inhibited when stored in CO_2 alone. *Staphylococcus aureus* was also tested on poultry meat. Although growth occurred in modified atmospheres (vacuum packs and 100% CO_2) at 22 °C, no enterotoxin was detected.

Table 3.5 The effect on pathogens of storage on modified atmosphere packaged meats at a variety of temperatures

Pathogen	Meat	Atmosphere and temperature (°C) of storage											
		VP			50% N_2 + 50% CO_2			80% O_2 + 20% CO_2			100% CO_2		
		0	5	12	0	5	12	0	5	12	0	5**	12
Aeromonas	Lamb	-	+*	ND	-	-	ND	-	-	ND	-	-	ND
	Beef	-	-	ND	-	-	ND	-	-	ND	-	-	ND
Escherichia coli	Beef	-	-	-	-	-	-	-	-	+	-	-	-
Listeria monocytogenes	Lamb	-	+/-	ND	-	+/-	ND	-	+/-	ND	-	-	ND
	Beef	-	-	ND	-	-	ND	-	-	ND	-	-	ND
*Salmonella enteritidis***	P**	ND	-	+	ND	ND	ND	ND	-	+	ND	-	-
typhimurium	Beef	-	-	+	-	-	-	-	-	+	-	-	-
Staphylococcus aureus+	P+	ND	-	+++	ND	ND	ND	ND	ND	ND	ND	-	+++
Yersinia enterocolitica	Lamb	+	+	ND	+/-	+	ND	-	+	ND	-	+/-	ND
	Beef	-	-	ND	-	-	ND	-	-	ND	-	-	ND

+	Growth
-	No growth
+/-	Growth in some cases (strain variation or growth in only some packs of meat)
ND	Experiment not done
*	*Aeromonas hydrophila* did not grow in VP at 5 °C, although *Aer. caviae* did grow.
**	Poultry inoculated with *Sal. enteritidis* was stored at 3 and 10 °C rather than 5 and 12 °C
+	Poultry inoculated with *Staph. aureus* was stored at 3 and 22 °C rather than 5 and 12 °C
++	*Staph. aureus* grew at 22 °C in VP and 100% CO_2 but no enterotoxin was formed even at this temperature

Discussion

There was a relatively high level of contamination of the beef prepared under supposedly hygienic conditions in the abattoir. The mesophilic aerobic count was $>10^4$ g^{-1} at the outset. At the time of spoilage the flora of the modified atmosphere packaged beef was dominated numerically by lactic acid bacteria. This is in accord with previous studies (Table 3.6). Huffman *et al.* (1975) reported an exception. They found a population consisting mainly of strictly aerobic bacteria on supposedly MAP meat. It is possible that their packs were imperfectly sealed. The effect of such a condition on the microbial flora on meat was noted in the present study (pp. 42). In the present study the numbers of *Brochothrix thermosphacta* increased on meat in atmospheres having low levels (<50%) of carbon dioxide. It was inhibited on meat stored in high levels of this gas (Sutherland *et al.*, 1977; Erichsen and Molin, 1981). The low numbers - less than 1% of the total population - of pseudomonads and Enterobacteriaceae were expected because the selective pressures exerted by the inhibitory atmosphere favour the growth of Gram positive organisms (Haines, 1933a; Clark and Lentz, 1976; Sutherland *et al.*, 1977; Erichsen and Molin, 1981). The exception in the present study was 80% O_2 + 20% CO_2 at 5 °C. In this case the numbers of both Gram positive and negative organisms

increased. There was a difference, however, in that the growth rate of Gram negative bacteria was much slower than that of the lactic acid bacteria and *Brochothrix thermosphacta*. The extent of retardation of growth of the Gram negative organisms is probably due to synergism between the carbon dioxide, low pH and low temperature. This combination is well known to prolong the lag phase and decrease the growth rate (e.g. Clark and Lentz, 1976).

Packaging meat in modified atmospheres certainly inhibited the growth of pathogens (Table 3.5). On high pH beef, however, *Yersinia* was found to grow at 30% the rate of the spoilage flora in CO_2 packs whilst *Aeromonas* and *Listeria* were also able to grow in vacuum pack (Gill and Reichel, 1989). In this study, the meat was of normal pH (<5.75) and the growth of all these pathogens was inhibited on beef. The growth of *E. coli* at 12 °C must be of concern regarding the potential for temperature abuse of packs. The growth of *Yersinia*, *Aeromonas* and *Listeria* was also noted on lamb in one or more of the atmospheres. The normal pH of lamb tends to be higher than that of beef, so the growth was not unexpected when the results of Gill and Reichel (1989) are considered (see above).

Even though the lactic acid bacteria grew quite extensively, the onset of meat spoilage was delayed in all atmospheres tested until ≥21 (5 °C) and ≥56 (0 °C) days. This is an appreciable extension of retail meat packs *vis-à-vis* those stored aerobically, these having a 5 and 10 day shelf-life at equivalent temperatures.

This overview shows that the MAP of beef steaks gave results similar to those of previous workers (Table 3.6 below and A1.1 in Appendix 1). It outlines the development of the microbial population such that further detailed analysis can be assessed in relation to the population as a whole.

Table 3.6 Modified atmosphere packaging of beef

Meat	Storage characteristics				Spoilage characteristics			Comments	Ref.
Cut	Temp. °C	Atmosphere	OTR[a] of film	Gas to meat ratio	Shelf life[b] (days)	Cause of spoilage[c]	Dominant organisms[d]		
Ribeye	-1	100% CO_2	N.S.	N.S.	<23	C	Aerobic, not		1
rolls		70% N_2 + 25% CO_2 + 5% O_2			<23		LAB[s]		
Roasts	1-3	VP	32		34	O	Lb[si]		2,3
		20% CO_2 + 80% N_2		1:1	34	O/C	Lb		
		20% CO_2 + 80% O_2			20	O/C			
		25% CO_2 + 25% O_2 + 50% N_2			20	O/C	Lb/Ps		
		51% CO_2 + 30% O_2 + 18% N_2 + 1% CO			34	O	Lb		
Steaks	1	75% O_2 + 25% CO_2	8.1	N.S.	13	App	Leu[i]		4, 5
		VP 28d then 75% O_2 + 25% CO_2			14+13	App			
		VP			70	N.D.			
	4								
Hung 7d	1	75% O_2 + 25% CO_2	<2-4	2:1	21-25	O/C	Leu[si]		6
VP 21d					35	O			
Hung 7d	6				11-13	O/C	Lb/Ps/B/Ent		
VP 21d					25	O			
Low pH	3	VP	2-4	N.S.	42	-	LAB[s]	Colour	7
		100% CO_2			>45	-		better with	
High pH		VP			34	O	LAB/Ent	storage in	
		100% CO_2			>45	-	LAB	CO_2	
Sirloin	1	75% O_2 + 25% CO_2	<10	2:1	18-22	O	LAB[s]		8
		VSP	<5		38	O			

a OTR = Oxygen transmission rate measured in ml m^{-2} 24 h^{-1} at 1 atmosphere. The temperature and relative humidity at the time of measurement varies with film data.

b Where possible time is taken from slaughter. The methods and times of ageing differ. Where retail shelf-life was tested, time is given as time in modifed atmosphere + time in retail display.

c - No spoilage noted, O Off-odour, C Discolouration, F Off-flavour, App. General appearance

d G-ve Gram negative organisms, LAB Lactic acid bacteria, B *Brochothrix thermosphacta*, Ps. *Pseudomonas* spp., Leu *Leuconostoc* spp., Ent Enterobacteriaceae, Lb *Lactobacillus* spp., Tested with [s] selective medium only, [i] identification of isolates from total counts or [si] selective media were used but isolates taken from total counts were also identified.

* VP Vacuum pack

Refs. 1, Huffman *et al.* (1975); 2, Christopher *et al.* (1979a); 3, Seideman *et al.* (1979a); 4, Hanna *et al.* (1981); 5, Savell *et al.* (1981); 6, Nortjé and Shaw (1989); 7, Taylor *et al.* (1990); 8, Rousset and Renerre (1991)

CHAPTER 4

LACTIC ACID BACTERIA IN THE MICROBIAL FLORA ON MODIFIED ATMOSPHERE PACKAGED BEEF STEAKS

INTRODUCTION

Members of the 'lactic acid bacteria' (LAB) are Gram-positive, catalase-negative asporogenous bacteria which produce lactic acid as the main (homofermentative) or an important (heterofermentative) product of carbohydrate fermentation. Currently this group includes the following genera (Aguirre and Collins, 1993): *Aerococcus, Carnobacterium* (Collins *et al.*, 1987), *Enterococcus* (Schleifer and Kilpper-Bälz, 1984), *Erysipelothrix, Gemella, Globicatella, Lactobacillus, Lactococcus* (Schleifer *et al.*, 1985), *Leuconostoc, Pediococcus, Streptococcus* (Schleifer and Kilpper-Bälz, 1987), *Tetragenococcus* and *Vagococcus* together with the newly described "*Weisella*" (Collins *et al.*, 1993).

It is now recognised that lactic acid bacteria are important members of many microbial associations in a diverse array of habitats. They are found in milk and associated products - the primary source of organisms used in the initial taxonomic studies of this group - raw meat and meat products, fermented vegetables, fermented and unfermented beverages, bread, pickles, in agriculture (silage and probiotics) and on/in man and animals as well as being used in industrial processes (lactic acid and dextran - a blood plasma substitute - production). It is well known that lactic acid bacteria are important also in the manufacture of many meat products, *viz.* starter cultures for fermented sausages and similar products. The adoption of modified atmospheres for the packaging of fresh meats has shown, moreover, that lactic acid bacteria become numerically dominant in such environments also. In MAP meats lactobacilli, leuconostocs and carnobacteria are of primary importance, but lactococci and pediococci occur also (Table 4.1).

Orla-Jensen (1919) studied in detail lactic acid bacteria from milk and dairy products. His approach to the taxonomy of micro-organisms was unique at the time of the study; he exploited the physiological attributes of micro-organisms in order to define genera and species. Three sub-genera - *Streptobacterium, Betabacterium* and *Thermobacterium* - were included in the genus *Lactobacillus* (Orla-Jensen, 1919). These groupings were maintained until the mid 80's in Bergey's Manual of Systematic Bacteriology (Sneath *et al.*, 1986) though not accepted as sub-genera. Group I (the thermobacteria) included the obligately homofermentative strains, group II the facultatively heterofermentative (streptobacteria) and group III the obligately heterofermentative ones - betabacteria (Kandler and Weiss, 1986). There have been many taxonomic changes, however, since the publication of this edition of the Manual. *Lactobacillus piscicola* and *Lact. divergens* were transferred to the new genus, *Carnobacterium* (Collins *et al.*, 1987). A phylogenetic analysis

Table 4.1 Lactic acid bacteria isolated from meat

Meat	Packing method[a]	Isolation medium[b]	Test methods[c]	No. of strains	Identification (%)	Authors[d]
Beef	VP	TSA MRS	B/M/API	177	10 *Leuconostoc mesenteroides* 65 Heterofermentative rods 25 Homofermentative rods	1
Meat	VP	PCA/AA MRS$^{5.5}$/BQ	B/M/API	100	31 Non-aciduric rods 57 *Lactobacillus sake/curvatus* 7 possibly *Leuconostoc paramesenteroides*	2
Meats/ products	-	Briggs MRS LBS AA	B/M/Ferm	690	2 *Leuconostoc* spp. 64 Streptobacteria, possibly *Lact. sake/curvatus* 18 Thermobacteria, some *Lact. acidophilus* 17 Betabacteria, *Lact. fermentum, viridescens, brevis*	3
Meat	VP radurised	mod MRS	B/M/Ferm	113	88 *Lact. sake* with 4 sub-groups, 7 *Lact. sake/curvatus*, 3 *Lact. curvatus*, 2 *Lact. farciminis*	4, 5[e]
Meat/ products	-	AA MRS	B/M/Ferm - miniaturised	229	*Lactobacillus* spp. only: 57 *sake*, 22 *curvatus*, 7 *divergens*, 3 *brevis*, *plantarum*, *viridescens*, 2 *hilgardii*, *camis*, *casei*, 1 *halotolerans*, *farciminis*, 0.5 *alimentarius*, *coryneformis*	6
Pork Beef	VP	AA MRS	B/M/Ferm	246	30 *Leuconostoc*, 13 *Lactococcus* 10 Heterofermentative lactobacilli (*Lact. divergens)* 47 *Lact. sake/curvatus*	7
Meats/ products	-	TGE	B/M/Ferm/ Ass	94	5 *Carnobacterium piscicola* 9 *Carnobacterium divergens* 10 *Lact.* sp. biovar 1, 18 *Leuconostoc* 4 *Lact.* sp. biovar 2, 12 *Lact.* sp. biovar 3 30 *Lact.* sp. biovar 4, 4 *Lact.* sp. biovar 5	8
Pork Chicken	MAP irradiated	BHIYE	API + 4 tests	94	84 *Lact.* possibly *sake*, 3 *Carnobacterium* sp. 2 *Lact.* possibly *curvatus*, 1 *Leu.* possibly *dextranicum* 4 Organisms similar to *Carnobacterium* 2 *Leuconostoc* sp.	9
Carcass	-	mod MRS	API + 1 test	35	Ten groups of ropy, slime-producing bacteria described. No species identification	10

a VP, Vacuum pack; MAP Modified atmosphere packs in a variety of atmospheres b TSA, Tryptone soy agar; MRS, de Man, Rogosa, Sharp; PCA, Plate count agar; AA, Acetate agar (Rogosa); MRS 5.5, MRS at pH 5.5; BQ see **Fournaud, J.; Salé, P. and Valin, C.** (1973) Conservation de la viande bovine sous emballage plastique sous vide ou en atmospheres controlées. Aspects biologiques et microbiologiques. In "*Proceedings of the 19th Meeting of European Meat Research Workers*". pp. 287-315, Paris; Briggs, Tomato juice agar; LBS, Lactobacilli selective agar from BBL; mod MRS, modified MRS, see Chapter 2 p. 45 (von Holy and Cloete, 1992); TGE, Tryptone glucose extract agar; BHIYE, Brain heart infusion yeast extract

c B, biochemical; M, morphological; API, API 50CH kit; Ferm, Carbohydrate fermentation tests; Ass, assimilation tests

d 1 Hitchener *et al.*, (1982); 2 Shaw and Harding (1984); 3 Morishita and Shiromizu (1986); 4 Hastings and Holzapfel (1987a); 5 Hastings and Holzapfel (1987b); 6 Schillinger and Lücke (1987a); 7 Schillinger and Lücke (1987b); 8 Borch and Molin (1988); 9 Grant and Patterson (1991a); 10 Mäkelä *et al.* (1992)

e Results from two studies were very similar (both 88% *Lact. sake*) so only one study has been included.

of 16S ribosomal RNA sequences compared over 50 lactobacilli species and related organisms (Collins *et al.*, 1991). Three groups containing the majority of lactobacilli strains were found; the paramesenteroides group, *Lb. casei/Pediococcus* and the delbrueckii group. The paramesenteroides group has since been transferred to a new genus "*Weisella*" with the description of "*W. hellenica*", a species isolated from Greek sausage (Collins *et al.*, 1993). The *Lb. casei/Pediococcus* group comprised 29 lactobacilli and five pediococci strains. This group also included *Lact. sake* and *Lact. curvatus*, both of which have been found to be very common in MAP meats (Table 4.1).

The lactic acid bacteria are particularly difficult to enumerate, isolate (see Chapter 2, pp. 44-50), characterise and identify. Indeed, the differentiation of the genus *Lactobacillus* was described in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994) in the following manner. "This large genus requires special expertise to identify the species; many test reactions are weak and dependent on the composition of the media and the exact cultural conditions." The descriptions given for the genus *Lactobacillus* (Kandler and Weiss, 1986) in Bergey's Manual of Systematic Bacteriology (Sneath *et al.*, 1986) have been found to give results disparate to those of type strains (Hastings and Holzapfel, 1987b; Borch and Molin, 1988). Of the 22 carbohydrate tests described in the Manual (Kandler and Weiss, 1986), 18% of the type strains used in studies with meat isolates were found to differ by at least five tests (Borch and Molin, 1988). It was surmised that this may have been due to the difference in the methods used for analysis, or differences in the original environments from which type and test strains were isolated. Reference strains, few of which were from meat or meat products, included in the characterisation of isolates often clustered separately from test strains (Hastings and Holzapfel, 1987a; Borch and Molin, 1988; Grant and Patterson, 1991a).

The media used and incubation conditions are an important factor in the results of any study of these organisms. Whittenbury (1963) determined the effect of different oxygen tensions, temperatures and salt concentrations on a wide range of lactic acid bacteria. A large difference between the optimum conditions of different strains was observed. Some strains required aerobic conditions for utilisation of particular substrates (*Lact. plantarum* for glycerol, mannitol and sorbitol), whilst others grew only in anaerobic and a few strains in microaerobic conditions. Certain species grew in all the oxygen concentrations pertaining in the soft agar medium. Temperature was found to affect the growth and oxygen requirements of particular micro-organisms. *Lactobacillus fructivorans* showed uniform growth throughout the range of oxygen concentrations at 30 °C but grew

only in diminished concentrations at 37 °C. There are a number of commercially available kits for the characterisation and identification of lactic acid bacteria. The bio-Merieux API 50 system was developed for medical and dairy isolates. The identification of meat isolates is not possible with the current data base. There is also a kit, produced by Biolog (3938, Trust Way, Hayward, California. 94545), based on a microtitre plate containing 95 tests which give a "global phenotype analysis" (Bochner, 1989). The Gram positive plate is recommended for lactic acid bacteria with the appropriate incubation medium. The data base contains all LAB species previously found in meats, other than *Carnobacterium* spp..

Lactobacillus sake is one of the most common isolates of lactic acid bacteria from fresh, chilled meat (Table 4.1). Large variations in the biochemical profile of this species have been noted. Champomier *et al.* (1987) found 13 biovars amongst the 84 strains identified with this species. Hastings and Holzapfel (1987a) also demonstrated eight clusters with different biochemical profiles in which *Lact. sake* was the most common isolate. In a similar study four clusters were designated *Lact. sake* and one *Lact. sake/curvatus* (Hastings and Holzapfel, 1987b). There is a problem distinguishing *Lact. sake* from *Lact. curvatus* (Hastings and Holzapfel, 1987a, b). The morphology of *Lact. curvatus* (presence of curved or horseshoe shaped cells) in comparison with *Lact. sake* (straight rods) proved to be unreliable; *Lact. curvatus* was not always curved and *Lact. sake* occasionally revealed this morphology (Hastings and Holzapfel, 1987a).

Increasingly, other methods have been adopted for the analysis of this difficult group (Table 4.2). In recent years, some workers have used a range of techniques based on molecular biology in an attempt to resolve taxonomic problems within the group. Indeed, various genera and species (Schleifer and Kilpper-Bälz, 1984; Schleifer *et al.*, 1985; Collins *et al.*, 1987; Schleifer and Kilpper-Bälz, 1987; Collins *et al.*, 1989; Martinez-Murcia and Collins, 1990; Collins *et al.*, 1991; Collins *et al.*, 1993; Martinez-Murcia *et al.*, 1993) have been defined by such methods. As yet little attempt has been made to amend or devise characterisation systems that could be used routinely. Many though would be useful as a confirmatory test rather than for routine identification.

Despite variations in the growth requirements of lactic acid bacteria the use of standard conditions for test and type strains should give comparable and, if analysed by numerical taxonomic methods, appropriate identifications. The methods adopted for the identification of lactic acid bacteria in this study were chosen from a review of the literature which sought to identify the important tests for the differentiation of lactic acid bacteria

Table 4.2 Developments in identification methods for lactic acid bacteria which followed on from simple morphological, biochemical tests and physiological tests

Method	Bacterial group analysed	Reference
Computer assisted identification	LAB	Döring *et al.*, 1988
Morphology (including SEM)	LAB	Dykes *et al.*, 1993b
Gas chromatographic analysis of fatty acids	LAB	Dainty *et al.*, 1984
	Atypical streptobacteria	Decallonne *et al.*, 1991
Gas liquid chromatography - metabolic end products	LAB	Thornhill and Cogan, 1984
Total soluble protein profiles	LAB	Dykes and von Holy, 1993
Plasmid profiles	*Leuconostoc*	Cavin *et al.*, 1988
	Lactobacillus curvatus and *sake*	Vogel *et al.*, 1991
	LAB	Dykes *et al*, 1993a
Probes - genus specific rRNA	*Leuconostoc*	Nissen *et al.*, 1993
	Carnobacterium	
- species specific DNA	LAB species	Lonvaud-Funel *et al.*, 1991
	Lact. curvatus	Petrick *et al.*, 1988
- species specific rRNA	*Lact. curvatus, sake,* and *pentosus/plantarum*	Hertel *et al.*, 1991
	Lactococcus spp.	Klijn *et al.*, 1991
	Leuconostoc spp.	
	Carn. divergens, mobile and *piscicola/gallinarum*	Brooks *et al.*, 1992
- *in situ* identification	*Lactococcus*	Beimfohr *et al.*, 1993
Ribotyping	*Lactobacillus* spp.	Rodtong and Tannock, 1993

species (Table 4.3). A relatively small number of morphological, physiological and fermentation tests were combined in a miniaturised form (where possible) in order to analyse a large number of isolates rapidly. Numerical taxonomic methods were employed such that organisms giving similar characterisation profiles clustered together. Some of the principles of numerical taxonomy were described by Sneath and Sokal (1973) *viz:*

1) "The greater the content of information in the taxa of a classification and the more characters on which it is based, the better a given classification will be.
2) *A priori,* every character is of equal weight in creating natural taxa.
3) Overall similarity between any two entities is a function of their individual similarities in each of the many characters in which they are being compared."

In the present study two methods of calculation were used to analyse the results, these being the "Jaccard" (J) and "Simple Matching" (SM) mathematical coefficients. The Jaccard co-effficient does not include results where the two strains being compared were negative for a particular test. The simple matching coefficient (Figure 4.3) gives equal weighting to all test results (positive matches, negative matches and tests where one of the two strains being compared gives a positive and the other a negative result). As the tests used are for

differentiation of isolates, both similarities and dissimilarities were considered important. Thus, although both were done for comparison, the simple matching coefficient was used in subsequent analysis and identifications. It must be stressed that the numerical taxonomic methods used in this study were used as an aid to identification rather than a means of studying the phylogenetic relationships.

Table 4.3 **Comparison of characteristics of lactic acid bacteria isolated principally from meats**

CHARACTERISATION	Tests used in the characterisation of lactic acid bacteria isolated from meats [1]																													
RESULTS	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t	u	v	w	x	y	z	A	B	C
TAXONOMIC STUDIES:																														
Lactococcus*[2] *lactis		+		v	-	v	+		+	-			-	+	+		v	-	-	-		-		+	+	-	v	+	v
garvieae		+		+	-	+	+		+	v			-	+	+		+	v	-	-		-		+	+	-	v	+	-
plantarum		-		+[w]	-	+	+		-	-			-	-	+		+	-	+	-		-		-	+	+	+	+	-
raffinolactis		-[+]		v	v	+	+		+	-			v	+	+		v	+	v	-		+		-[+]	+	-	-	+	+
***Carnobacterium*[3]**																														
divergens		+	-	+	-		+	+		+[-]		-	-		+	+	-		v	-	-	-	-	+	+	-	+	+	-
gallinarum		+	-	+	-		+	+	-	+		-	-	-	+	+	-		+	+	-	-	-	+	+	-	+	+	+
mobile		+	-	-	-		+	+	+	-		-	+		+	+	-		-	-	-	-	-	+	+	-	+	+	-
piscicola		+	-	+	-		+	+	+	+		-	+	+	+	+	+	-	+	+	+	-	-	+	+	-	+	+	-
Leuconostoc*[4] *gelidum				+	+	+	+	+	-	-	-	-		-	v	+	-	+	-	+		+	-	v	+	-	+	+	+
mesenteroides				∓	+	-	∓		+					∓	+	+		+		v		±		-	∓				±
carnosum				-	-	-	-		-	-	-			-	-	±	-	∓	-	+		-	-	v	-	-	+	+	-
TEST STRAINS:																														
***Lactococcus* spp.[6]**		-	-		d		+		+	d	d		-	d	+		+	-	d			-	-	d	+	d	+	+	-
***Carnobacterium*[6]**																														
divergens		+	+		-		+		-	v				-	+		-	-	v			-	-	+	+	-	+	+	-
***(Lact. divergens)*[5]**					-	+			-	v	±		-	-	+		-	v	∓			-	-	±	+	-	±	+	-
Cluster 2[7]		+	-		-		+	+	-		-			-	+	+	-	-				-	-		+	-	+	±	-
***divergens/piscicola*[8]**			-	+	-	+	+	+	+	v	±	-	-	-	+	+	v	-	∓	∓	-	-	-	+	+	-	v	+	-
***piscicola*[6]**		+	-		-		+		v	-				v	+		+	v	-			v	-	+	+	-	+	+	-
Cluster 1[7]		+	-		-		+	+	+		±			+	+	+	+	v				-	-		+	±	+	+	-
***Leuconostoc* spp.[6]**		-	+		v		v		v					v	v		v	v	v			v	v	v	v	v	v	v	v
[9]	D	-	+		+			+						-	+	+		+					+		+	-		+	+	+
[10]	D	-	+	-	+	+	+		v	-	-	-	-	v	+	+	-	v	-	+	-	v	-	+	v	-	+	+	+
Cluster 4[8]			-	-	-	-	-	+	+	v	-	-	-	-	v	+	-	-	-	+	-	-	-	-	-	-	+	+	-
Cluster 6[8]			v	-	v	-	-	+	+	v	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	v
Cluster 9[7]		-	±		-		±	+	∓		-			-	+	±	-	-				-	-		-	-	+	+	-

Table 4.3 contd. **Comparison of characteristics of lactic acid bacteria isolated principally from meats**

CHARACTERISATION RESULTS	Tests used in the characterisation of lactic acid bacteria isolated from meats																													
	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t	u	v	w	x	y	z	A	B	C
Lactobacillus sake[6]		v	-		v		v		+	+				-	-		-	+	-			-	-	+	v	-	+	+	-
[11]				v	±	v	v	+	+	+	v	-	-	∓	v	+	-	+	-	-		-	-	+	v	-	+	+	-
Cluster 1[8]			∓	∓	v	v	v	+	+	v	-	-	-	∓	∓	+	-	+	-	∓	-	-	-	v	v	-	+	+	-
[5]		±	-	v			v		+	+	-		-	∓	∓		-	+	-			-	-	+	v	-	+	+	-
Cluster 4[12]		-	-	v	v		∓	+	+	+	+	-	+	v	v	∓	-	+	-	v		v	∓	+	v	-	+	+	
Cluster I[13]	DL			v	+		v	+	+	+	v		v	v	v	v	-	+	-	v		v	v	+	v	-	+	+	
Lact. sake/curvatus																													
Cluster 1[12]		-	-	∓	v		v	+	+	+	+	-	+	v	∓	v	-	+	-	v		v	v	+	v	-	+	+	
Cluster 2[12]		-	-	-	+		v	+	+	±	±	-	±	∓	v	v	-	v	-	v		∓	∓	+	±	-	v	±	
Cluster 3[12]		-	-	∓	±		∓	+	±	±	±	-	±	∓	v	-	-	±	-	-		v	+	+	v	-	+	+	
Cluster II[13]	DL			-	+		+	+	+	+	+		+	∓	-	+	-	-	-	∓		∓	∓	+	+	-	±	+	
Lact. sake/farciminis																													
Cluster 5[12]		-	-	v	v		-	v	+	+	+	-	+	v	v	∓	-	v	v	+		v	-	+	+	-	+	+	
Lact. farciminis																													
Cluster IV[13]	L			-	-		v	-	v	v	+		+	v	v	v	-	-	-	v		-	-	v	+	-	+	+	
Lact. curvatus[6]		v	-		-		v		+					-	+		-	-	-			-	-	+	v	-	v	v	-
[5]		v	-		∓		v		v	v	∓		-	∓	±		-	-	-			-	-	+	v	-	v	v	∓
Cluster III[13]	DL			-	+		-	+	+	v	+		+	-	+	-	-	-	-	v		-	-	+	+	-	-	+	
Cluster 3[8]			v	-	-	-	-	+	+	v	-	-	-	v	v	+	-	-	-	-	-	-	-	+	-	-	-	-	-
Streptobacteria																													
Cluster 1[9]	DL	-	-	+	-	+	+	+	+		±	-	-	-	-	+	-	-	-	+	-	-	-	+	+	-	±	+	-
Cluster 2[9]	DL	+	-	-	-	-	-	+	+		-	-	-	-	-	+	-	+	-	-	-	-	-	+	-	-	∓	+	-
Cluster 3[9]	DL	+	-	±	+	+	+	+	+		-	-	-	-	-	+	-	+	-	-	-	-	-	+	+	-	+	-	-
Cluster I[10]	DL	+	-	+	v	+	+		v	v	+	-	-	v	+	+	v	v	v	v	-	-	-	+	+	-	+	+	-
Cluster II[10]	DL	v	-	v	v	v	v		+	+	-	-	-	v	v	+	-	v	-	-	-	-	v	+	v	-	+	v	-
Group S6[14]	DL	-		+	-		+		+					v	v		-	v	-			-	-	+	+	-	v	+	-
Group S7[14]	DL	-		v	+		+		+					+	v		-	+	-			v	-	+	+	-	+	v	-

Table 4.3 contd. **Comparison of characteristics of lactic acid bacteria isolated principally from meats**

CHARACTERISATION	Tests used in the characterisation of lactic acid bacteria isolated from meats																												
RESULTS	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t	u	v	w	x	y	z	A	B	C
Betabacteria																													
Cluster 4[9]	L	+	+	+	-	+	+	+	-		-	-	-	-	+	+	-	-	+	-	-	-	-	+	+	-	+	+	-
Cluster 5[9]	L	+	+	+	-	+	+	+	±		+	-	-	±	+	+	+	∓	-	+	-	-	-	+	+	-	+	+	-
Group B3[14]	DL	+		-	+		-	+	+						-	+	v	-	+				-	-	+	-	-	-	+
***Lactobacillus* spp.**																													
Cluster 4[7]		-	-		-		±	+	+		-			∓	+	+	-	-				-	-		+	-	∓	v	-
Cluster10[7]		-	-		-		-	+	+		-			+	±	+	-	-				-	-		±	-	-	±	-
Cluster11[7]		v	-		±		-	+	+		-			∓	∓	+	-	+				-	-		-	-	+	v	-
Cluster12[7]		v	-		-		v	+	+		-			-	∓	+	-	±				-	-		-	v	+	+	-
Cluster 14[7]		+	-		-		∓	+	+		-			-	-	+	-	-				-	-		-	-	+	+	-

1 Tests:- a, isomer of lactic acid produced; b, ammonia production from arginine; c, gas production from glucose; d-z and A-C fermentation tests; d, amygdalin, e, L-arabinose; f, arbutin; g, cellobiose; h, fructose; i, galactose; j, gluconate; k, glycerol; l, inositol, m, inulin; n, lactose; o, maltose; p, mannose; q, mannitol; r, melibiose; s, melezitose; t, methyl-D-glucoside; u, methyl-D-mannoside; v, raffinose; w, rhamnose; x, ribose; y, salicin; z, sorbitol, A, sucrose; B, trehalose; C, D-xylose

2 Schleifer *et al.* (1985)
3 Collins *et al.* (1987)
4 Shaw and Harding (1989)
5 Schillinger and Lücke (1987a)
6 Döring *et al.* (1988)
7 Borch and Molin (1988)
8 Grant and Patterson (1991a)
9 Hitchener *et al.* (1982)
10 Shaw and Harding (1984)
11 Champomier *et al.* (1987)
12 Hastings and Holzäpfel (1987a)
13 Hastings and Holzapfel (1987b)
14 Morishita and Shiromizu (1986)

* % of positive strains
+ 90-100
± 76-89
v 25-75
∓ 11-24
- 0-10

MATERIALS AND METHODS

Lactic acid bacteria were isolated from beef steaks stored in MAP either inoculated with pathogens or uninoculated. These experiments have been described previously (Chapter 3).

Isolations were made from the trial of beef steaks inoculated with *Listeria monocytogenes*. Storage of the plates at Leatherhead before delivery and subsequent isolation at Bath led to loss of cell viability. Thus, although 1171 isolations were attempted from this trial, the number of strains successfully sub-cultured was too limited to warrant their further characterisation.

Bacteria were isolated from APT (incubated anaerobically - Oxoid gas generating kit) used for the enumeration of LAB from modified atmosphere packaged beef steaks. The trial involved the inoculation of beef steaks with *Salmonella typhimurium* and subsequent packaging in MAP. The packs were stored at 5 and 12 °C and sampled at regular intervals (see Chapter 3 pp. 70-72 for further details). Lactic acid bacteria were isolated from uninoculated steaks stored at 5 °C only; previous work had shown little difference between uninoculated and inoculated steaks and isolations from all other trials done at 0 or 5 °C.

In the following section all figures are % w/v unless otherwise stated.

Isolation

Colonies were picked randomly by drawing a line across the bottom of the Petri dish and selecting the five closest to the line. Cells were transferred to Whittenbury's basal medium (WH) with glucose (0.5): 0.5 yeast extract, 0.5 bacteriological peptone, 0.5 lab-lemco, 0.05 v/v Tween 80 and 1.2 agar for the solid medium (Whittenbury, 1963) and streaked twice to ensure purity. Media were incubated anaerobically (Oxoid gas generating kit) at 25 °C for up to four days.

Maintenance

Cells from single colonies on WH agar were transferred to WH broth (*ca* 3.5 ml) in bijou bottles and incubated at 25 °C until turbidity was observed (1 - 3 days). For long term storage, an equal volume of sterile glycerol was added to each bijou (Schillinger and Lücke, 1987a) and homogenised before storage at -20 °C. When required for use, 50 µl of broth with glycerol were transferred to a bijou containing the WH broth and incubated until

growth was evident. Cells were streaked onto WH agar (+ glucose) to ensure purity prior to characterisation. The type strains of *Lactobacillus sake* (NCFB 2714), *Lact. curvatus* (NCFB 2739), *Lact. bavaricus* (NCFB 2588), *Leuconostoc carnosum* (NCFB 2776), *Leuc. mesenteroides* ssp. *mesenteroides* (NCFB 523), *Leuc. gelidum* (NCFB 2775), *Carnobacterium divergens* (NCFB 2763), *Carn. mobile* (NCFB 2765) and *Carn. gallinarum* (NCFB 2766) were maintained in a similar manner. Strains from the Bath University culture collection were also used. These had been previously identified and included strains of: *Lact. fructivorans, Lact. plantarum* and *Brochothrix thermosphacta.* Previously identified strains of *Lact. sake* strain 706, *Lactococcus lactis* (2 strains), *Pediococcus pentosaceus* (3 strains), *Ped. acidilactici* were kindly provided by Campden Food and Drink Research Association.

Characterisation

Preliminary characterisation was based on the Gram stain and catalase test. Gram positive, catalase negative organisms were used in further tests. The cell morphology was recorded.

Preparation of the microtitre plate

Twenty two carbohydrates were used in a miniaturised (microtitre plate) method (Jayne-Williams, 1976). The WH basal medium (no glucose) was prepared, with the addition of 0.0024% bromocresol purple (BCP), in concentrated (X1.3) form. In other words, the substrates required for 100 ml of medium were dissolved in 75 ml distilled water, but with allowance for the addition of the stock solution of carbohydrate. Thus, for normal use, 65 ml of water was used. The basal medium was autoclaved (121 °C for 15 min). Each carbohydrate was made up as a 10X stock solution (5%) and filter sterilised. The required volume was added to the cooled, sterile basal medium before dispensing (150 µl) into the wells of a microtitre plate. The carbohydrates used in the analysis were: amygdalin, L-arabinose, arbutin, cellobiose, galactose, lactose, maltose, mannose, mannitol, melibiose, α methyl-D-glucoside, raffinose, ribose, salicin, sorbitol, sucrose, trehalose, turanose, D-xylose. Glucose was used as a positive control, and water containing no carbohydrate as a negative control.

Other tests

The production of gas from glucose was tested by the method of Gibson and Abd-el-Malek (1945) according to the description in Harrigan and McCance (1976): Yeast extract 0.25, D-glucose 5.0, manganese sulphate 0.0004, skim milk powder 10.0, nutrient broth 0.5, agar 0.24. The nutrient agar was made up (dissolved and heated to dissolve the agar) at 20% of the volume required and the remaining ingredients were combined in distilled water and warmed in a steamer. The medium was distributed in *ca* 7 ml volumes in test tubes and sterilised by steaming for 30 minutes on each of three successive days. When required, the medium was melted by steaming, then cooled to 45 °C and inoculated with a culture from storage in a bijou. The broth was thoroughly mixed and cooled before sterile, melted nutrient agar was added to the top of the medium to a depth of approximately 2-3 cm.

The presence of arginine dihydrolase was examined in the medium of Hitchener *et al.* (1982): yeast extract 0.5, tryptone 0.5, K_2HPO_4 0.2, glucose 0.05, DL-arginine hydrochloride 0.6, Tween 80 0.1 v/v, $MnSO_4.4H_2O$ 0.005. The medium was made up in concentrated form (X1.3) sterilised (in an autoclave at 121 °C for 15 min) and dispensed into microtitre plates (150 µl per well).

Inoculation

Cells from WH agar were transferred with a wooden applicator stick to ca. 2 ml of sterile distilled water in vessels used with a multipoint inoculator (Denley A400). Fifty µl was removed and dispensed into each carbohydrate medium in the microtitre plate and into one well of the arginine dihydrolase medium (separate plate). To determine growth on various media, the cell suspension remaining in the vessels was used to inoculate AA, STAA, APT, H_2O_2 using the multipoint inoculator. Production of H_2O_2 was determined using APT modified by the addition of ABTS and horseradish peroxidase (Marshall, 1979).

Incubation

All tests were incubated at 25 °C. The carbohydrate tests and the solid media were incubated anaerobically (Oxoid gas generating kits). The arginine test was incubated aerobically. All tests (except gas from glucose) were monitored regularly and scored after three days unless further incubation was necessary. The tubes showing production of gas from glucose were observed after two, four, seven and 14 days.

Recording the results

Carbohydrate fermentation tests were scored as positive if a colour change from purple to yellow had occurred (Figure 4.1). The wells were always compared to positive and negative controls to ensure validity of a recording. Hydrogen peroxide production was scored after 30 min. exposure of the test medium to air at room temperature. Blackening or greening of the agar around colonies was scored as positive. With the arginine test, Nessler's reagent (1 drop) was added to each well and an orange colour recorded as positive. Gas production from glucose was recorded as positive when bubbles trapped beneath the agar plug or in the skim milk medium were evident. Growth on AA, STAA and APT was scored as positive.

Biolog Gram positive plates

These were used according to the manufacturer's instructions (Biolog, Hayward; USA). Strains were grown on BLA agar (Biolog) for 24 or 48 h. Cells were removed from the agar by rolling a pre-moistened sterile cotton swab across its surface. The swab was twirled against the edge of a tube containing 18-20 ml BLA suspension medium (Biolog) to form a uniform suspension. One hundred and 50 µl were used to inoculate each well of a "Gram positive (GP)" microplate, which had been equilibrated at room temperature before use. Microplates were incubated anaerobically (Oxoid gas generating kit) at 25 °C overnight. Plates were incubated for a further 24 hours where necessary.

Analysis of results

The characteristics of lactic acid bacteria were analysed with appropriate methods of numerical taxonomy using the PHYLIP (Phylogeny Inference Package) programmes (Joseph Felsenstein, Dept. Of Genetics SK-50, University of Washington, Seattle, WA 98195, USA). Test results were scored as positive (1) or negative (0) and the similarity between strains was calculated using the simple matching or Jaccard coefficients. The strains were clustered using an unweighted pair group method (UPGMA).

Reproducibility

Ten % of the strains in each trial were re-tested to determine the reproducibility of the tests. The following equation was used to give a reproducibility percentage:

$$\frac{\text{Number of tests giving the same reaction}}{\text{Total number of tests}} \times 100$$

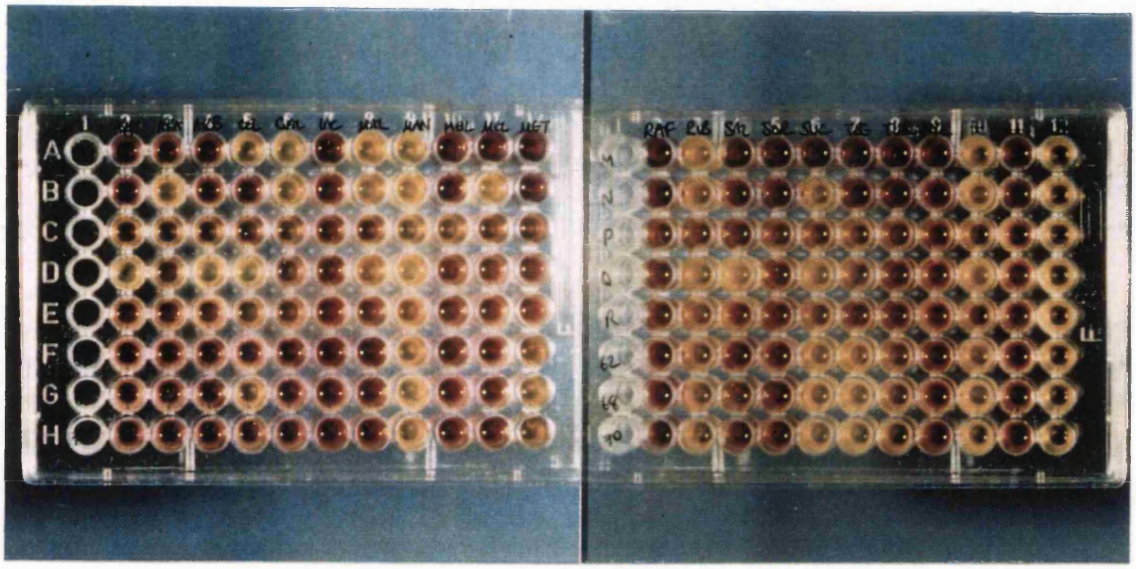


Figure 4.1 **Characterisation of lactic bacteria using a microtitre plate method**

M, *Leuconostoc gelidum*, N, *Lactobacillus sake*, P, *Brochothrix thermosphacta*; Q, *Carnobacterium. divergens*; R, *Leu. carnosum*; 62, 68, 70 Meat isolates

Amy, Amygdalin; Ara, Arabinose; Arb, Arbutin; Cel, Cellobiose; Gal, Galactose; Lac, Lactose; Mal, Maltose; Man, Mannose; Mol, Mannitol; Mel, Melibiose; Met, Methyl-D-glucoside; Raf, Raffinose; Rib, Ribose; Sal, Salicin; Sor, Sorbitol; Suc, Sucrose; Tre, Trehalose; Tur, Turanose; Xyl, Xylose; +, Glucose; -, No substrate

RESULTS

As would be expected from previous studies (Table A1.1, Appendix 1) lactic acid bacteria tended to predominate on MAP beef steaks inoculated with *Salmonella typhimurium* or uninoculated control steaks stored at 5 °C (see Chapter 3, pp. 70-72). The results presented in this section are from the uninoculated meat only.

Four hundred and 91 bacteria were isolated from APT incubated anaerobically during the five weeks of storage of MAP at 5 °C. Many of these were *Brochothrix thermosphacta* [as identified by the cell morphology, catalase reaction and ability to grow on STAA (Table 4.4)].

Table 4.4 Micro-organisms from beef steaks stored at 5 °C in MAP and isolated from APT incubated anaerobically

Micro-organism	Number	% of total micro-organisms	% of lactic acid bacteria
Carnobacterium divergens	154	32.8	56.8
mobile	7	1.5	2.6
Lactobacillus spp.	12	2.6	4.4
sake/curvatus	63	13.4	23.2
Leuconostoc gelidum	16	3.4	5.9
mesenteroides	15	3.2	5.5
Unidentified LAB	4	0.9	1.5
Brochothrix thermosphacta	149	31.8	-
Others	27	5.8	-
LAB which failed to grow on sub-culture	22	4.7	-
TOTAL	469*	100	100

* Figure excludes 22 strains from day 3, not included in the analysis as only isolates from VP were available

The lactic acid bacteria were characterised by traditional biochemical and carbohydrate tests, adapted to microtitre plates where possible. As described in the Materials and Methods (pp. 92), the sugar fermentation tests were compared with the positive (glucose) and negative (no carbohydrate) controls. On occasions, pH drift in the negative control was noted, and the positive recording of a test required a significant difference in colour to the negative control. Some strains grew very weakly in the medium used in the test. Thus, the tests were compared with the glucose control to ensure adequate growth before recording the results. Tests were repeated where necessary. The results of the characterisation were clustered according to the numerical taxonomic methods described previously. The results are shown for both the Jaccard (Figure 4.2) and simple matching (Figure 4.3) coefficients. The patterns of clusters distinguished with the two coefficients were very similar. The Jaccard coefficient tends to show groups having

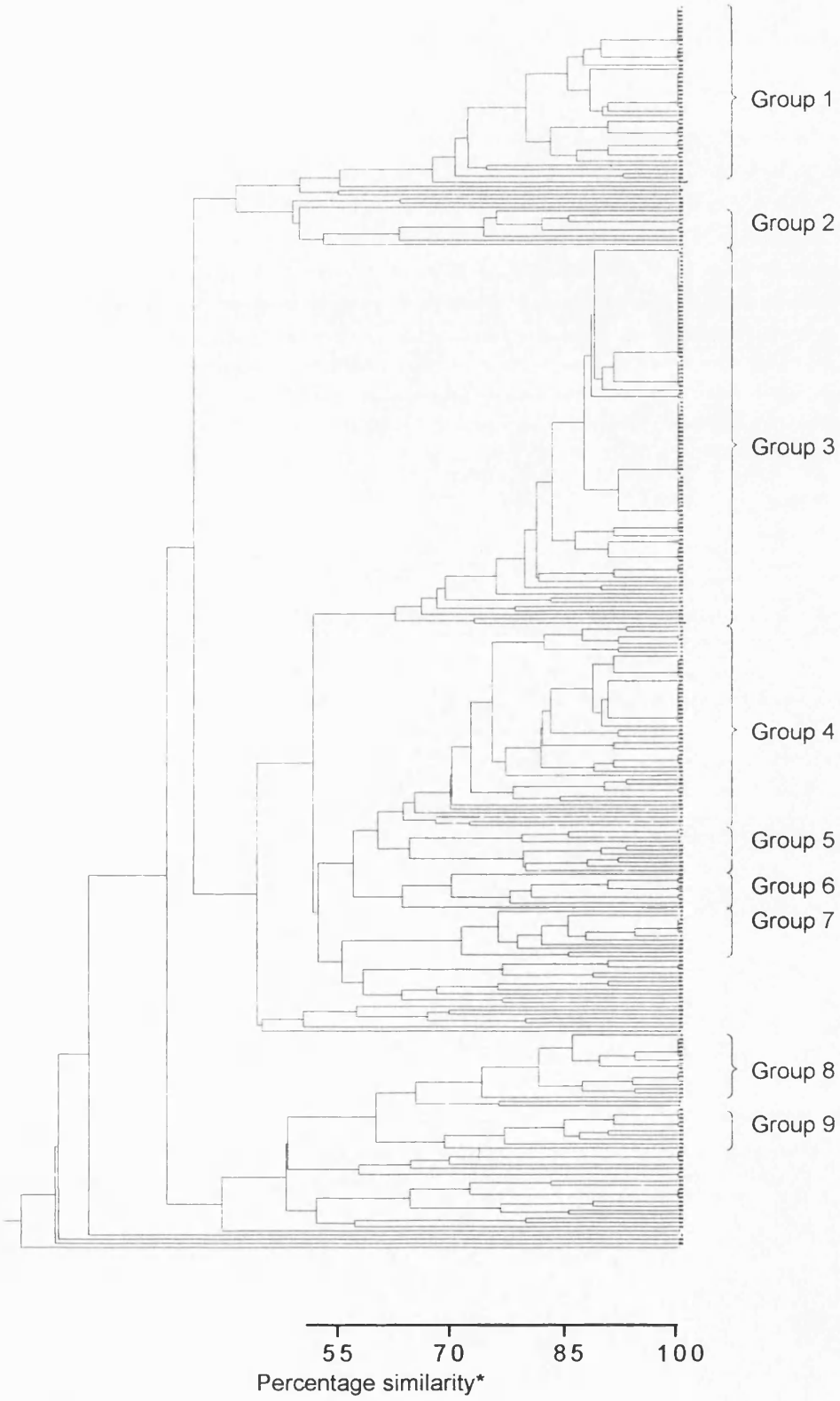


Figure 4.2 **Lactic acid bacteria isolated from the Salmonella II trial, characterised by traditional methods and clustered by the Jaccard co-efficient**

*The scale is approximate, based on the percentage similarity calculated from the similarity co-efficients between pairs of strains. The calculation is an arithmetic mean and exact numbers cannot therefore be specified.

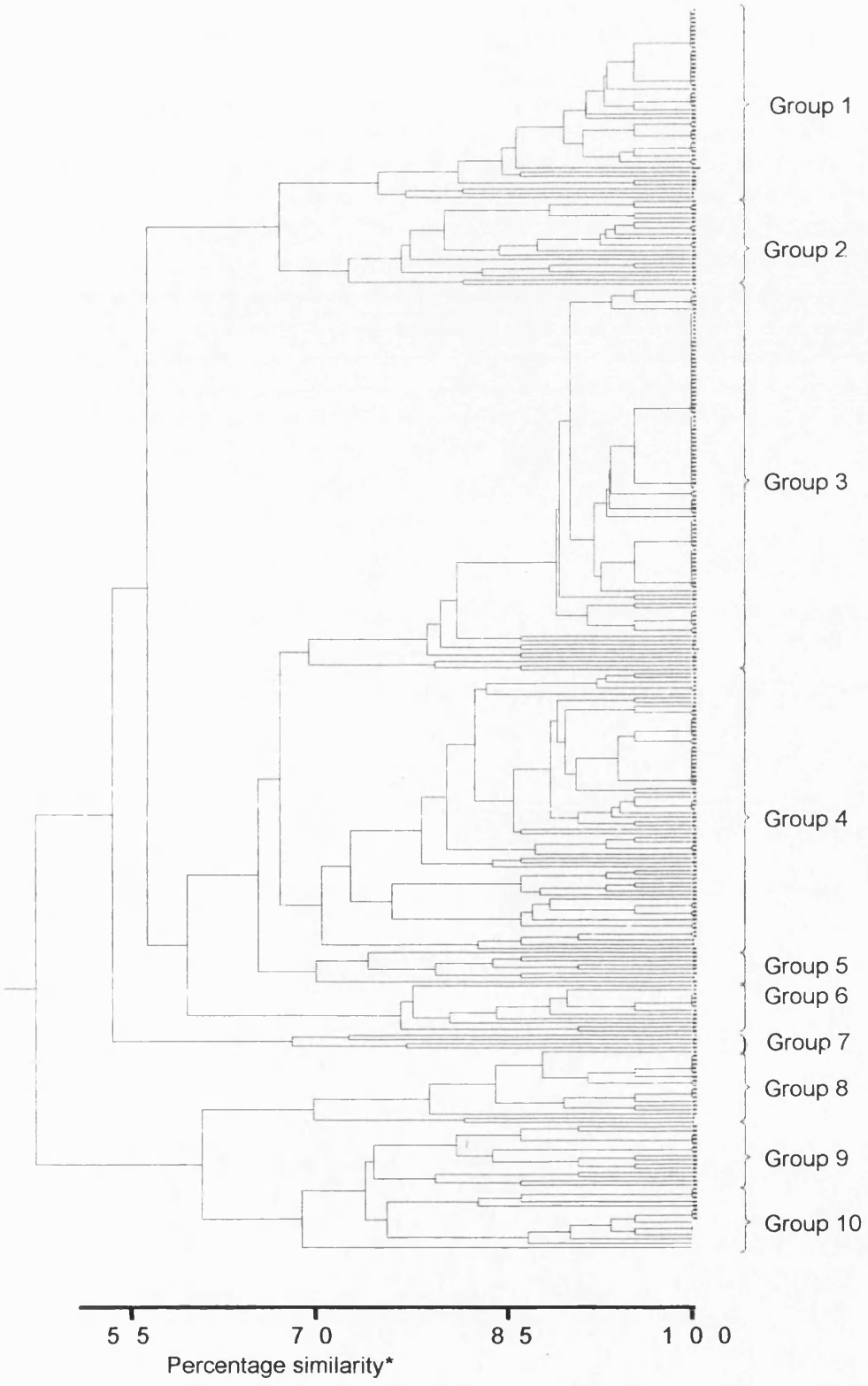


Figure 4.3 **Lactic acid bacteria isolated from the Salmonella II trial, characterised by traditional methods and clustered by the simple matching co-efficient**

*The scale is approximate, based on the percentage similarity calculated from the similarity co-efficients between pairs of strains. The calculation is an arithmetic mean and exact numbers cannot therefore be specified.

lower similarity than those analysed with S_{SM}. This may be due to the calculations used in the two methods. In the S_j analysis, only dissimilarities and positive matches are used in the calculation whereas negative matches also are used in S_{SM}.

Ten groups were evident with the simple matching coefficient (Figure 4.3) using a boundary of approximately 70% similarity:

Groups 1 and 2

These two groups contained 63 meat strains and four previously identified strains. Group 1 contained two type strains (*Lactobacillus sake* and *Lact. curvatus*), strains of *Lact. sake* and *Brochothrix thermosphacta* identified previously, together with 42 of the meat isolates. Group 2 contained the remaining 21 strains. *Lactobacillus sake* and *Lact. curvatus* are difficult to distinguish (Hastings and Holzapfel, 1987a, b). In this study the two groups were therefore identified with *Lact. sake/curvatus*.

Groups 3 and 4

Carnobacterium divergens and a *Broch. thermosphacta* strain were present in group 3 together with 91 lactic acid bacteria from the MAP meat. Group 4 contained 63 field strains. The two groups were identified with *Carn. divergens*.

Group 5

The *Carn. mobile* type strain clustered with seven meat strains. The profile of the group closely matched that described by Collins *et al.*, (1987). These strains were therefore identified with *Carn. mobile*.

Group 6

This group contained 12 meat isolates. All of the strains were rod-shaped and 92% were able to grow on acetate agar. They were therefore described as *Lactobacillus* spp.

Group 7

There were only four isolates in this group, all of them being test strains. There was much variation between the strains, with 12/22 tests giving variable (16-84%) reactions (Table 4.5). None of the tests was positive for all strains. These organisms remain unidentified.

Group 8

The type strains of *Leuconostoc mesenteroides* and *Leuc. gelidum* together with 16 meat isolates were contained in this group. The characteristics of the group most closely matched those of *Leuc. gelidum* and the strains were therefore identified with this species.

Groups 9 and 10

Both groups 9 and 10 contained 15 strains, with those in group 10 including the type strain of *Leuc. carnosum*. The profile of this organism (Table 4.5) did not match that of either group (Table 4.3). The profiles of isolates in this group most closely matched that of *Leuc. mesenteroides* (Table 4.3) and they were therefore identified with this species.

Table 4.5 **Characteristics of each group of lactic acid bacteria from modified atmosphere packaged beef steaks, calculated from the simple matching coefficient**

		Tests used for the differentiation of lactic acid bacteria																						
Group	Total	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	46	$+^{100}$	$-^{0}$	$-^{2}$	v^{50}	$-^{4}$	$-^{4}$	$+^{98}$	$-^{0}$	$-^{7}$	$+^{98}$	$-^{0}$	$+^{91}$	$-^{0}$	$-^{2}$	$+^{100}$	$-^{9}$	$-^{0}$	$+^{96}$	$+^{93}$	$-^{13}$	$-^{0}$	$+^{93}$	v^{30}	$+^{96}$
2	21	$+^{100}$	$-^{0}$	v^{33}	v^{19}	$-^{14}$	$+^{81}$	$+^{95}$	$-^{5}$	v^{43}	$+^{95}$	$-^{10}$	$-^{5}$	$-^{5}$	$-^{0}$	v^{24}	v^{52}	$-^{0}$	$-^{0}$	$+^{95}$	$-^{5}$	$-^{5}$	$-^{14}$	$-^{0}$	$+^{90}$
3	92	$+^{99}$	v^{40}	$+^{86}$	$-^{4}$	$+^{99}$	$+^{100}$	$-^{5}$	$-^{3}$	$+^{98}$	$+^{99}$	$-^{0}$	$-^{0}$	$-^{0}$	$-^{0}$	$+^{98}$	$+^{100}$	$-^{0}$	$+^{98}$	$+^{91}$	v^{30}	$-^{1}$	$+^{95}$	$-^{3}$	$-^{2}$
4	63	v^{67}	$-^{0}$	v^{65}	$+^{94}$	$+^{98}$	v^{71}	$+^{86}$	v^{38}	$+^{100}$	$+^{100}$	v^{76}	v^{24}	$-^{0}$	v^{19}	$-^{0}$	$+^{87}$	$-^{6}$	$+^{98}$	$+^{90}$	$-^{3}$	$-^{6}$	v^{19}	$-^{0}$	$-^{2}$
5	14	$+^{100}$	$-^{0}$	$-^{7}$	v^{64}	v^{43}	$+^{93}$	v^{50}	v^{443}	$+^{93}$	$+^{100}$	v^{21}	v^{36}	$-^{7}$	v^{43}	v^{36}	v^{79}	$-^{7}$	$+^{86}$	$+^{86}$	$-^{14}$	$-^{0}$	v^{50}	$-^{7}$	$-^{0}$
6	12	$+^{100}$	$-^{0}$	$+^{100}$	v^{83}	$+^{92}$	$+^{100}$	$+^{100}$	v^{75}	$+^{100}$	$+^{100}$	v^{83}	$+^{100}$	$-^{8}$	$-^{8}$	v^{83}	$+^{100}$	$-^{8}$	v^{17}	$+^{100}$	v^{16}	$+^{100}$	v^{75}	v^{25}	$+^{92}$
7	4	v^{25}	v^{25}	v^{25}	v^{25}	v^{25}	$-^{0}$	$-^{0}$	v^{25}	v^{25}	v^{50}	$-^{0}$	$-^{0}$	v^{25}	$-^{0}$	$-^{0}$	v^{25}	$-^{0}$	v^{50}	$-^{0}$	$-^{0}$	$-^{0}$	v^{50}	$-^{0}$	$-^{0}$
8	18	$-^{0}$	$+^{94}$	$+^{100}$	$+^{100}$	v^{83}	$+^{100}$	$+^{94}$	$-^{0}$	$+^{94}$	$+^{94}$	v^{33}	v^{83}	$+^{88}$	$+^{100}$	$+^{93}$	$+^{93}$	$-^{0}$	$+^{100}$	$+^{100}$	$+^{100}$	v^{78}	$-^{11}$	v^{83}	$-^{6}$
9	15	$-^{0}$	$+^{93}$	$-^{0}$	$+^{100}$	$-^{0}$	$+^{100}$	$-^{0}$	$-^{0}$	v^{73}	$-^{13}$	$-^{0}$	v^{33}	$-^{7}$	v^{33}	$+^{87}$	$-^{7}$	$-^{0}$	$+^{100}$	v^{47}	v^{47}	v^{67}	$-^{7}$	v^{20}	$-^{0}$
10	15	$-^{0}$	$+^{93}$	$-^{40}$	$+^{93}$	$-^{0}$	$+^{93}$	$-^{0}$	$-^{0}$	v^{67}	$-^{7}$	$-^{0}$	v^{40}	$+^{100}$	$+^{93}$	$+^{87}$	$-^{0}$	$-^{0}$	$+^{100}$	v^{73}	v^{67}	$-^{0}$	$-^{7}$	v^{33}	$-^{0}$

Groups: Groups 1 and 2, *Lactobacillus sake/curvatus*; Groups 3 and 4, *Carnobacterium divergens*; Group 5, *Carn. mobile*; Group 6, *Lactobacillus* spp.; Group 7, Unidentified; Group 8, *Leuconostoc gelidum*, Groups 9 and 10, *Leuc. mesenteroides*

Tests: 1) Cell morphology (rod = +, coccus = -); 2) Production of CO_2 from glucose; 3-21) Fermentation of: 3) amygdalin; 4) arabinose; 5) arbutin; 6) cellobiose; 7) galactose; 8) lactose; 9) maltose; 10) mannose; 11) mannitol; 12) melibiose; 13) methyl-D-glucoside; 14) raffinose; 15) ribose; 16) salicin; 17) sorbitol; 18) sucrose; 19) trehalose; 20) turanose; 21) xylose; 22) Production of hydrogen peroxide

Percentage of posotive tests:

+ 85-100%

- 0-15%

v 16-84%

Most of the micro-organisms present initially on the beef steaks and isolated from APT incubated anaerobically were Gram positive (Table 4.6). The largest single microbial type, however, was the Gram negative, catalase positive rods (35%).

Table 4.6 Composition of the microflora isolated from beef steaks at the time of packaging after enumeration on APT incubated anaerobically

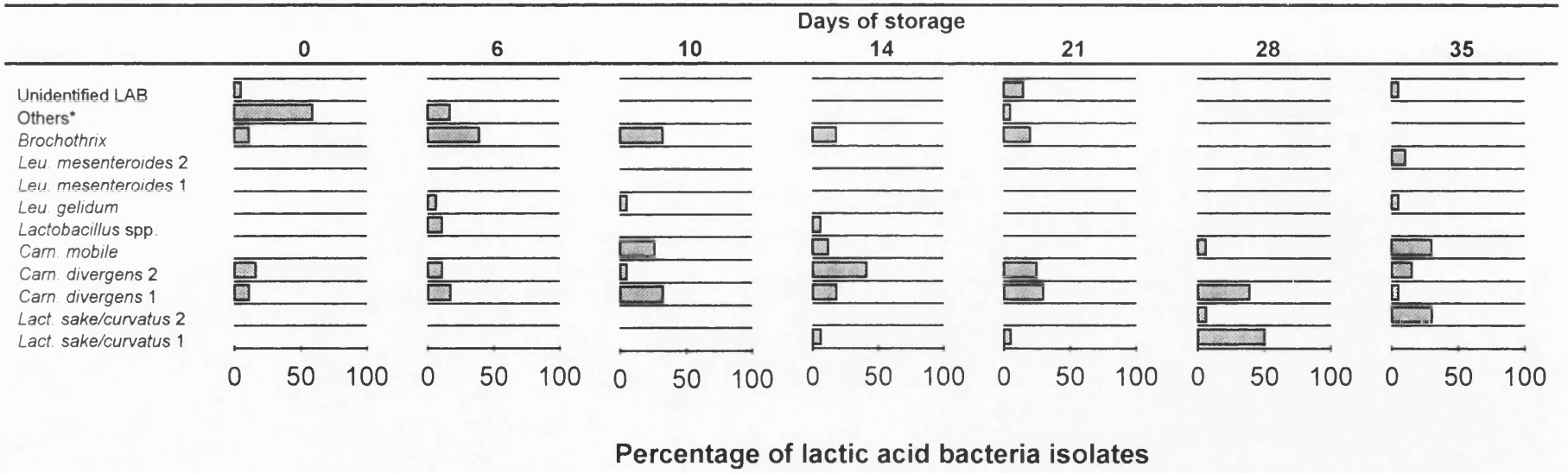
Type of micro-organism			% of initial microflora
Gram reaction	Cell morphology	Catalase reaction	
Negative	Rods	Positive	35
Positive	Cocci	Positive	24
Positive	Rods	Positive	12
Positive	Rods/Cocci	Negative	24
N.A.	Yeast	N.D.	6

N.A. Not applicable
N.D. Not determined

Figure 4.4 shows the changes in APT isolates during storage of MAP beef steaks. Numbers of *Brochothrix thermosphacta* (Gram positive rods able to grow on STAA) increased during the early stages of the storage in all modified atmospheres. The atmosphere containing CO_2 alone was the most inhibitory, with the percentage dropping to zero after two weeks storage at 5 °C. In vacuum packs and the 50% N_2 + 50% CO_2 atmosphere the numbers declined after three weeks. The atmosphere containing high oxygen, however, stimulated the growth of *Broch. thermosphacta* such that they dominated the population to the exclusion of other organisms by the end of storage (day 35).

The atmospheres containing 'high' levels of CO_2 (VP, 50/50 and the 100% CO_2) all showed a similar trend. Initially the only LAB species present was *Carn. divergens.* By the end of storage, however, there was a diverse LAB flora with 7 groups represented in the VP and CO_2 alone and 8 groups in the 50/50 atmosphere. In contrast, the 80/20 atmosphere was completely dominated by *Brochothrix thermosphacta.*

Leuconostoc spp. tended to be present in the populations towards the end of storage in all atmospheres, except 80/20 where the only isolation of members of this genus was after 14 days at 5 °C. This was not the case with *Carn. divergens* and *Carn. mobile* which were present throughout the study, with greater numbers in the early stages of storage. *Lactobacillus sake* tended to be isolated in low numbers, with the exception of the 100% CO_2 atmosphere. By the end of storage *Lact. sake* was about 35% of the population isolated from APT in CO_2 alone.

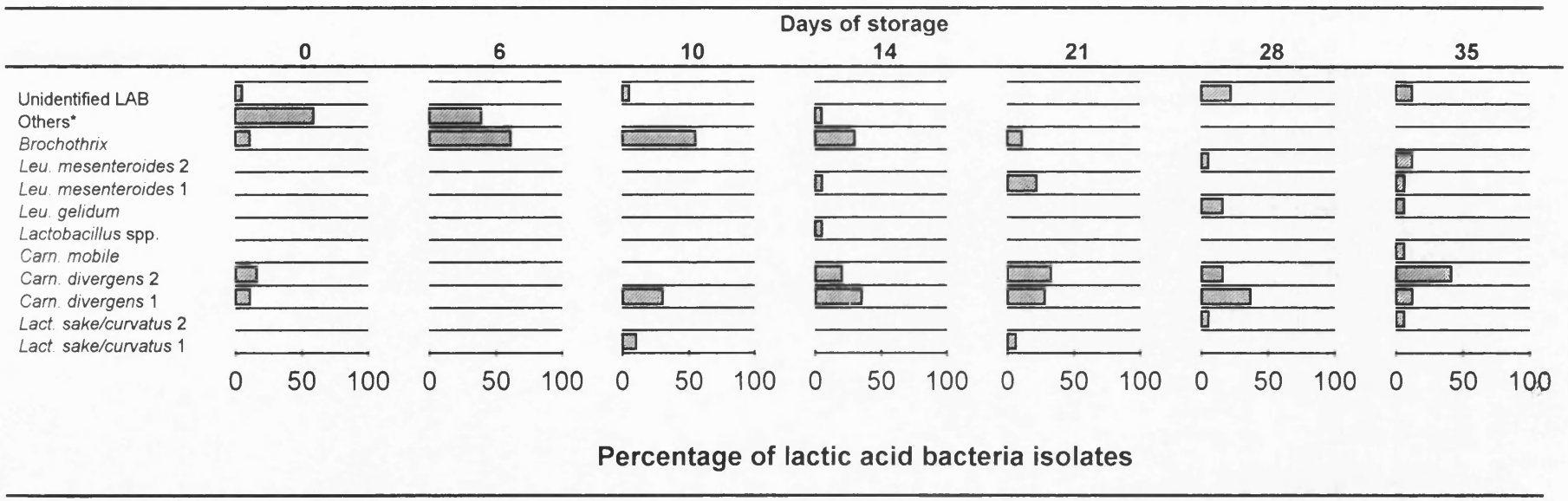


Reproducibility of test method - 94.0%

* Others (includes Gram negative rods; Gram positive, catalase positive cocci, and yeast. *Brochothrix - Brochothrix thermosphacta*

a) Vacuum pack

Figure 4.4 **Changes in the lactic acid bacteria population during storage of modified atmosphere packaged beef steaks stored at 5 °C**
Identification relates to the clusters determined by the simple matching co-efficient (Figure 4.3)

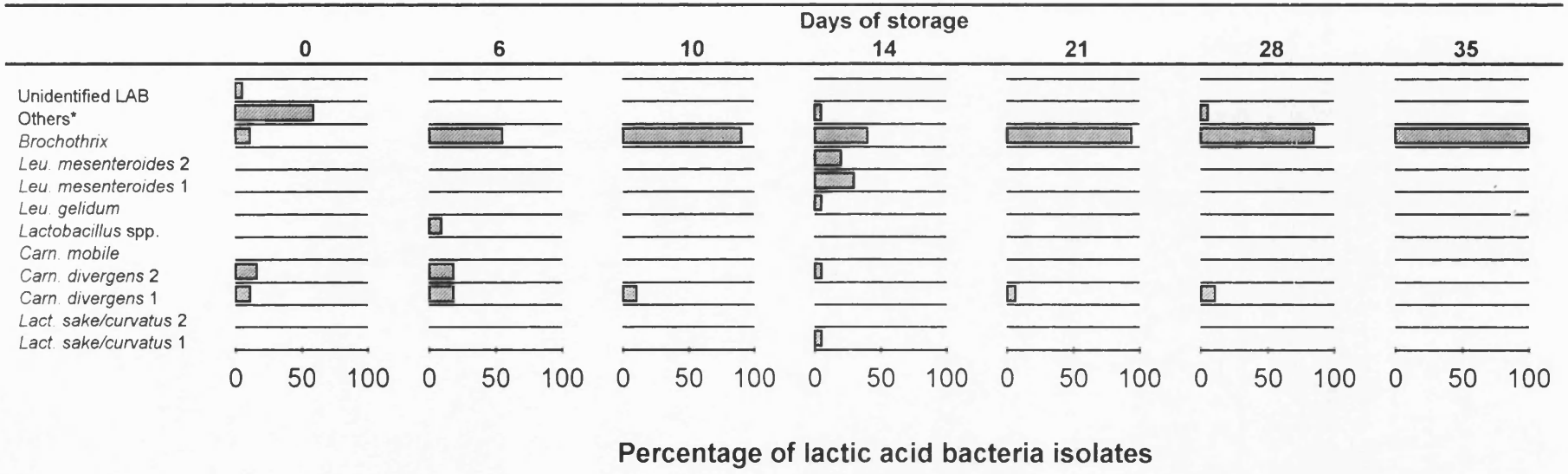


Reproducibility of test method - 94.0%

* Others (includes Gram negative rods; Gram positive, catalase positive cocci, and yeast. *Brochothrix - Brochothrix thermosphacta*

b) 50% N_2 + 50% CO_2

Figure 4.4 contd. **Changes in the lactic acid bacteria population during storage of modified atmosphere packaged beef steaks stored at 5 °C**
Identification relates to the clusters determined by the simple matching co-efficient (Figure 4.3)

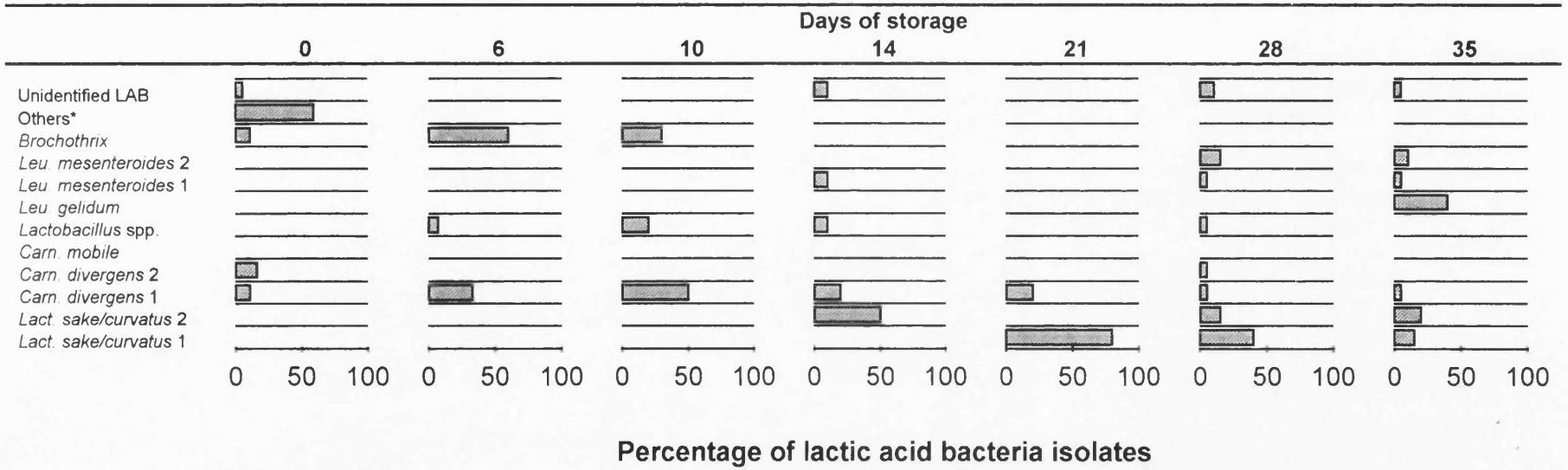


Reproducibility of test method - 94.0%

* Others (includes Gram negative rods; Gram positive, catalase positive cocci, and yeast. *Brochothrix - Brochothrix thermosphacta*

c) 80% O_2 + 20% CO_2

Figure 4.4 contd. **Changes in the lactic acid bacteria population during storage of modified atmosphere packaged beef steaks stored at 5 °C**
Identification relates to the clusters determined by the simple matching co-efficient (Figure 4.3)



Reproducibility of test method - 94.0%

* Others (includes Gram negative rods; Gram positive, catalase positive cocci, and yeast. *Brochothrix - Brochothrix thermosphacta*

d) 100% CO_2

Figure 4.4 contd. **Changes in the lactic acid bacteria population during storage of modified atmosphere packaged beef steaks stored at 5 °C**
Identification relates to the clusters determined by the simple matching co-efficient (Figure 4.3)

Biolog

A random selection of ten percent of the strains from the Salmonella trial (randomly chosen) were sub-cultured before use in the Biolog kit. The manufacturer's instructions advised growth on Biolog BLA medium before inoculation into the microtitre plate. Unfortunately several strains did not grow on this medium. It was often difficult also, to determine whether the test results were positive or negative due to extremely faint colour change. Tests often took longer than the manufacturer's recommended incubation times.

All strains (those that showed a colour reaction with glucose, the positive control test) were negative for α-cyclodextrin, inulin, mannan, D-arabitol, L-fucose, D-galacturonic acid, m-inositol, 3-methyl glucose, α-methyl D-mannoside, L-rhamnose, sedoheptulosan, D-sorbitol, xylitol, acetic acid, ß-hydroxybutyric acid, γ-hydroxybutyric acid, p-hydroxyphenyl acetic acid, α-keto-glutaric acid, lactamide, D-lactic acid methyl ester, L-lactic acid, D-malic acid, L-malic acid, mono-methyl succinate, propionic acid, succinamic acid, N-acetyl L-glutamic acid, alaninamide, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-glutamic acid, glycyl-L-glutamic acid, L-pyroglutamic acid, L-serine, putrescine, 2, 3, butanediol, thymidine-5'-monophosphate, uridine-5'-monophosphate, glucose-1-phosphate, glucose-6-phosphate and D-L-α-glycerol phosphate.

The Biolog system for "Gram positive" plates includes a separate data base for LAB which requires utilisation of the BLA medium. It was noted that the LAB data base did not include the genus *Carnobacterium*. This implies the use of an alternative medium for growth. In this case, however, all tests were done using the BLA medium. The Biolog data base was not used. Instead, the results were clustered in the same way as the more traditional test methods (using PHYLIP computer programme for numerical taxonomy). As before, both the Jaccard and the Simple Matching coefficients were used in the calculations. There were some similarities between the clusters (Figure 4.5 and 4.6) with two groups containing many of the same organisms. There were, however, only two groups observed with S_j, whilst there appeared to be three clusters present after the simple matching coefficient had been used to calculate the similarity. The groupings from the S_{SM} analysis did not match the results seen with the traditional methods. For instance, strain 371 (group 8 in the traditional analysis) was found in the Biolog identification to be in a group containing seven group 1 isolates and two group 3 isolates from the traditional results. This strain is a coccus and would therefore be expected to cluster with *Leuconostoc* spp.

(Groups 8, 9 and 10 in the traditional methods) rather than lactobacilli and carnobacteria. The identification of organisms based on this commercial kit was not used in further studies.

Genetic methods may be used to ascertain the accuracy of clustering on the basis of genotypic rather than phenotypic tests. An attempt was made to develop a method suitable for the identification of strains by cpmparison of the band patterns produced by restriction enzyme digests of ribosomal DNA (Mufti, 1994). Some of the strains isolated during the present study were used. Growth and subsequent isolation of rDNA were occasional problems. The results were analysed by the simple matching coefficient and UPGMA. Related type strains clustered in different groups whilst unrelated ones were present in the same group, *viz. Lact. sake* and two *Leuconostoc* type strains clustered together (Mufti, 1994).

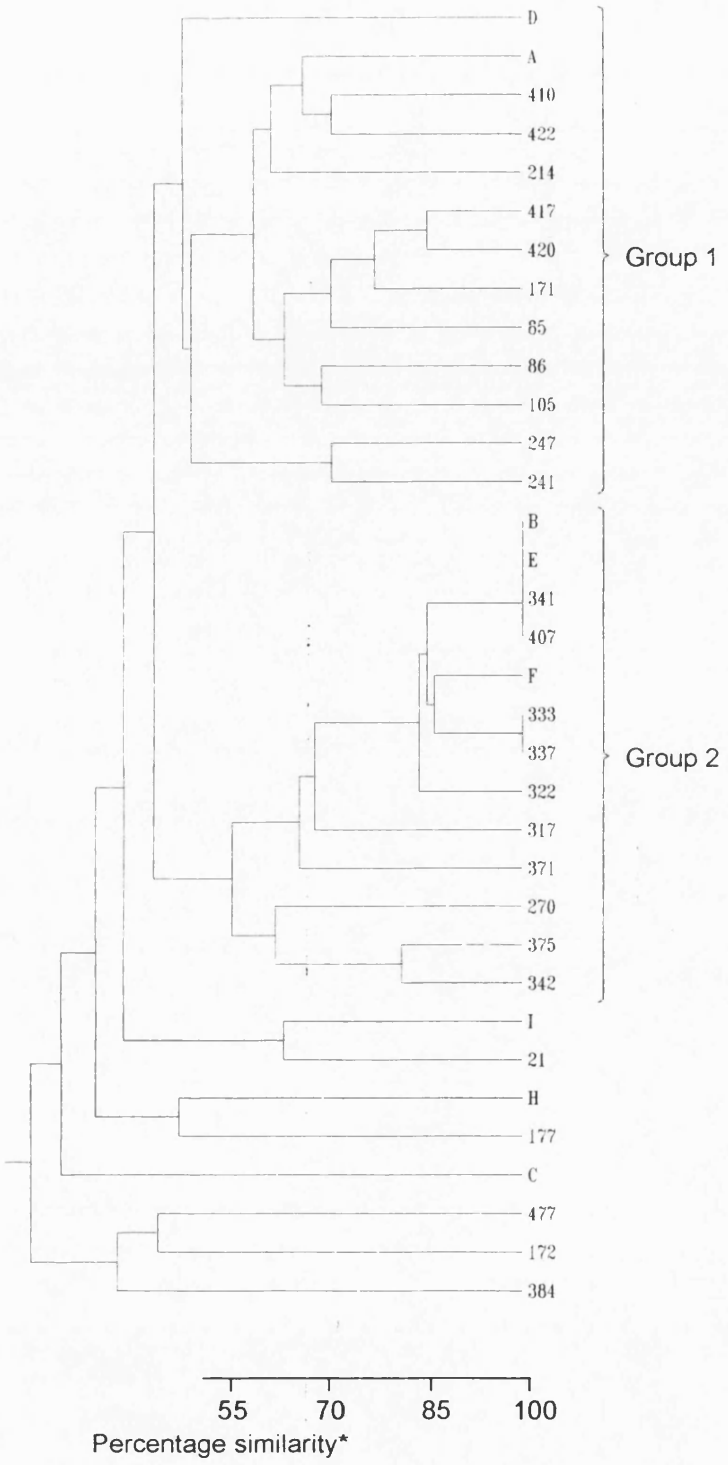


Figure 4.5 **Lactic acid bacteria isolated from the Salmonella II trial, characterised by the Biolog Gram positive microplates and clustered using the Jaquard coefficient**

*The scale is approximate, based on the percentage similarity calculated from the similarity coefficients between pairs of strains. The calculation is an arithmetic mean and exact numbers cannot therefore be specified.

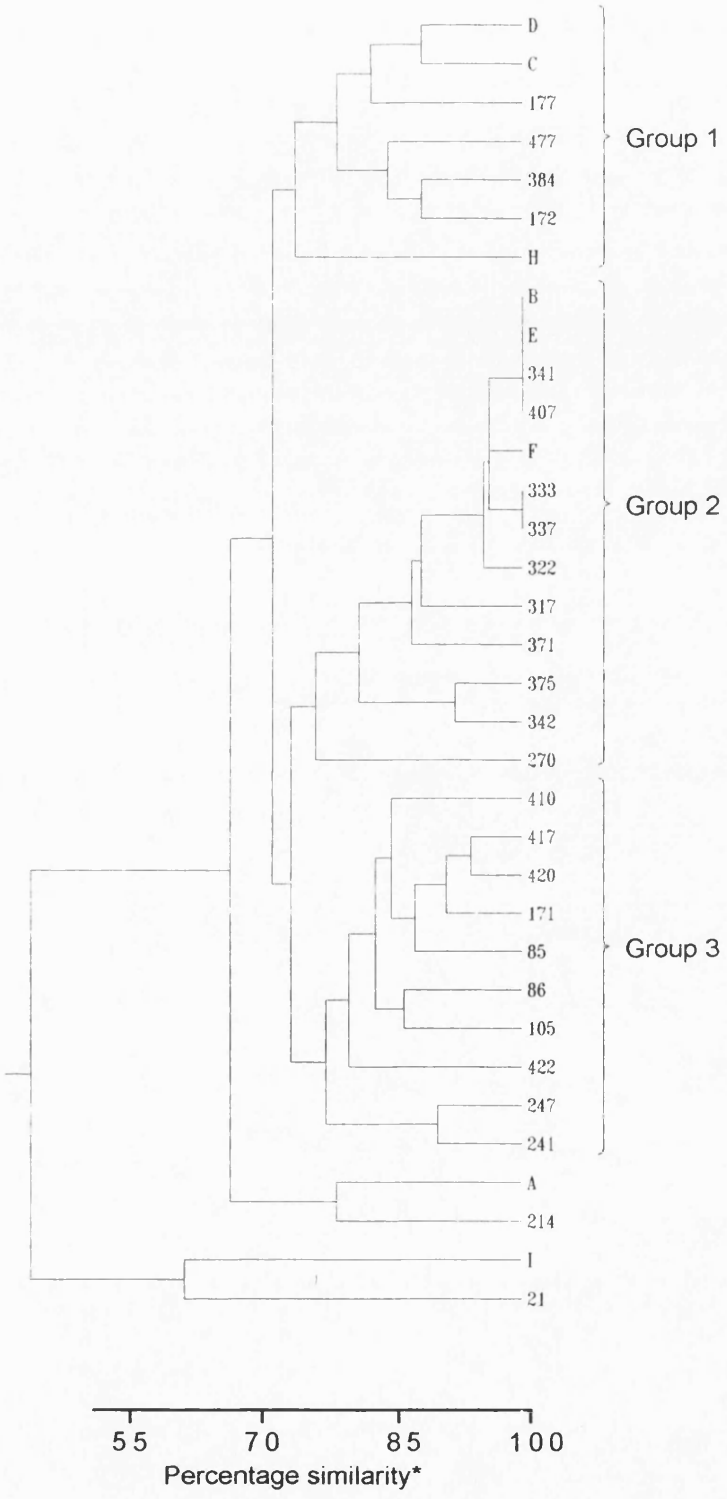


Figure 4.6 **Lactic acid bacteria isolated from the Salmonella II trial, characterised by Biolog Gram positive microplates and clustered using the simple matching coefficient**

*The scale is approximate, based on the percentage similarity calculated from the similarity coefficients between pairs of strains. The calculation is an arithmetic mean and exact numbers cannot therefore be specified.

DISCUSSION

As noted in the introduction there are problems with the identification of members of the LAB because of the heterogeneity of this group. Recent work using rRNA homologies as a molecular chronometer (Collins *et al.*, 1991) has helped to clarify the phylogeny of genera of LAB. Appropriate phenotypic characteristics for the differentiation of the LAB genera and species have not been determined, however, and identification of large numbers of lactic acid bacteria therefore remains a problem (Board and Jones, 1992). This can be partially overcome by the use of a combination of traditional methods for the characterisation of the micro-organisms together with numerical taxonomy for clustering as an aid to subsequent identification. It is important to include type strains in this form of analysis to ensure that members of clusters can be identified. This approach has been used successfully in previous studies (Borch and Molin, 1988; Dykes *et al.*, 1994; Grant and Patterson, 1991a; Hastings and Holzapfel, 1987b; Shaw and Harding, 1984; Shaw and Harding, 1989). The methods used in the present study were successful in the characterisation of 271 lactic acid bacteria. There were occasions when colour changes in the control wells in the microtitre plate were noted. A drift in pH of a negative control (no added carbon source) was probably due to the limited growth of some strains on the nutrients in yeast extract and peptone. Tests were scored as positive only if there was a significant difference in colour between the positive (glucose addition) and negative controls. Some strains only grew weakly in the positive control. In this case, the tests were only scored as positive if there was a distinct difference between the colour of the test and the negative control.

It is usual to determine the reproducibility of the characterisation methods by re-testing ten percent of the strains, chosen at random. In this trial, ten percent of strains including representatives from each of the groups, was re-tested not only with the microtitre plate methods but also with the commercially available Biolog kit. This kit did not work effectively. Some strains did not grow in the medium recommended for growth of inocula for tests, other strains did not grow in the medium in the microtitre plate. It has been used previously for Gram positive organisms (Ternström *et al.*, 1993). These authors found that more than 85% of isolates (mostly *Bacillus* spp.) gave false positive results because of the oxidation of the redox indicator.

In the present study on beef steaks packed in modified atmospheres (Salmonella trial), *Carnobacterium divergens* was found to be prevalent during the early stages of

storage. By the end of the study (35 days) a more varied LAB microflora was present. This was in contrast to the pseudomonad and Enterobacteriaceae results. The Gram negative flora tended to be diverse initially with only one or two species present at the end of the storage period. These findings may be related to the initial level of contamination of these organisms. As noted in the literature review (pp. 9-10) Gram negative organisms, particularly pseudomonads, are prevalent in the environments of abattoirs and meat processing factories. The meat surface is contaminated by large numbers relative to those of the LAB and the incidence of a varied flora at the time of packaging is not unlikely. The inhibitory environment in MAP meats (high CO_2 and low pH) may cause death or sub-lethal injury to most of the population. Only the best adapted were able to survive such that relatively few organisms persisted to the end of storage. Conversely, the unfavourable environment of meat production facilities leads to low numbers of LAB giving a smaller sample from which to isolate bacteria. Any bacteria present on the meat at the time of packaging may be sub-lethally stressed also, such that growth on elective media does not occur.

Although *Carn. divergens* was numerically dominant in the Salmonella II trial, other experiments in this study gave contrasting results. Of the LAB isolated on a variety of selective and elective media (Chapter 2 and Appendix 2), *Lact. sake* was the numerically dominant. This was true also of the LAB isolated from minced rump of beef acidified with acetic acid and vacuum packed (Appendix 3). *Lactobacillus sake/curvatus* comprised 100% of the LAB on the highly acidified meat. In the slightly acidified meats leuconostocs were found to be a small portion of the flora but in the control meat (water addition only) these organisms made up 75% of the microflora. The remaining 25% was *Carn. divergens.*

There have also been a number of other studies highlighting the LAB species important in meats (Table 4.1). Schillinger and Lücke (1987b) found that the initial microflora of LAB greatly influenced the subsequent composition of the population. *Lactobacillus sake/curvatus* was found to be numerically important (47%) in pork/beef (VP) with heterolactic rods (identified with *Lact. divergens* but subsequently renamed as *Carn. divergens*) forming 10% and leuconostocs 30% of the population (Schillinger and Lücke, 1987b). Hitchener *et al.* (1982) identified heterofermentative rods as the dominant (65%) organisms in vacuum packed beef. Homofermentative rods were also important (25%), with the remaining organisms (10%) being leuconostocs.

Survival and more importantly growth of the LAB species will be determined by the presence of organic acids (particularly lactic and acetic) and low pH as well as temperature,

composition of atmosphere and water activity. Although organic acids are known to be inhibitory to the micro-organisms, the mechanism of action is not understood (Baird-Parker, 1980; Brown and Booth, 1991; Cherrington *et al.*, 1991, Shelef, 1994). Indeed, the acidification of meat in this study (Appendix 3) altered the composition of the microflora dramatically (*Lact. sake* dominated in highly acidified meats and leuconostocs were prevalent in the control meat with water addition only). It is considered these lipophilic acids diffuse across the cell membrane in an undissociated form and dissociate within the cell causing a drop in cytoplasmic pH. Lactobacilli, however, are known to have a relatively high tolerance to low cytoplasmic pH. Some have been shown to survive intracellular pH of 4.4, relating to an external pH of 3.5 (Kashket, 1987; Nannen and Hutkins, 1991). The development of *Lact. sake* on meat acidified with acetic acid is, therefore, not unexpected.

The concentration of acetic acid in MAP meats has been proposed as an indicator of microbial spoilage (Kakouri and Nychas, 1994). In that case, it was attributed to the metabolic activities of *Broch. thermosphacta* or to members of the lactic acid bacteria. A similar increase in the concentration of acetic acid was noted during incubation of a meat juice medium inoculated with LAB. This was attributed to the heterofermentative metabolism of lactate by leuconostocs and carnobacteria (Drosinos, 1994). In glucose depleted conditions some lactobacilli have been shown to exhibit heterofermentative metabolism (Borch *et al.*, 1991). Thus, production of acetic acid by the lactic acid bacteria is likely to influence the development of the microflora.

The development of LAB on MAP meat will be affected by the factors already described. Antimicrobial agents such as bacteriocins may also be important and are known to be produced by species present in MAP meats (Table 4.7). Indeed, Vogel *et al.* (1993) suggested that a strain of *Lact. curvatus* became numerically dominant in fermented sausage by the production of curvacin A. A derivative of the strain without the ability to produce bacteriocin was unable to gain the same competitive advantage. Bacteriocins usually affect closely related micro-organisms (Table 4.7). The effect of these peptides on the LAB population in a meat environment is unknown, but their activity may be affected by meat constituents such as pH, fat content, temperature and presence of enzymes (Nettles and Barefoot, 1993). It has been suggested that bacteriocins disrupt the proton motive force, this would cause difficulties for the cells in maintaining the intracellular pH.

Table 4.7 Bacteriocin production from LAB isolated from meat and fish

LAB species	Bacteriocin	Source of strain	Inhibitory spectrum
Lactobacillus			
sake	Sakacin A	Meat	leuconostocs, lactobacilli, enterococci, *Listeria*
	M	Dry fermented sausage	leuconostocs, lactobacilli, carnobacteria, *Listeria*, *S. aureus*
	P	Dry fermented sausage	lactobacilli, leuconostocs, carnobacteria, enterococci, *Brochothrix*
	B*	Greek dry sausage	lactobacilli, leuconostocs, streptococci
	Lactocin S	Dry fermented sausage	pediococci, lactobacilli, leuconostocs
curvatus	Curvacin A	Meat	lactobacilli, leuconostocs, carnobacteria, micrococci, *Listeria*, staphylococci
Leuconostoc			
mesenteroides	Mesenterocin S	Cheese	*Listeria, Ent. faecalis, Bac. linens, Ped. pentosaceus*
gelidum	Leucocin A	Meat stored in 30% CO_2	leuconostocs, lactobacilli, pediococci, *Ent. faecalis*, *Listeria*
paramesenteroides	Leuconocin S	Lamb	*Lact. sake*, *Listeria*, *S. aureus*, *Y. enterocolitica, Aer. hydrophila*
carnosum	Carnocin	VP Vienna sausage	lactobacilli, carnobacteria, enterococci, pediococci, leuconostocs, *Listeria*
Carnobacterium			
piscicola	A1, A2, A3	VP meat	LAB
	B1, B2	VP meat	LAB
	Carnocin U149	Fish	lactobacilli, pediococci, carnobacteria
	Un-named	Beef	*Listeria*

Data from Nettles and Barefoot (1993) and * Samelis *et al.* (1994)

CHAPTER 5

ENTEROBACTERIACEAE IN THE MICROBIAL FLORA ON MODIFIED ATMOSPHERE PACKAGED BEEF STEAKS

INTRODUCTION

The enumeration of Enterobacteriaceae on selective media has shown that certain genera of this family are significant, but not dominant, members of the microbial associations on meats stored aerobically (Blickstad *et al.*, 1981; Dainty *et al.*, 1985) and more particularly in modified atmospheres (Dainty *et al.*, 1979; Blickstad *et al.*, 1981; Lee *et al.*, 1985; Nortjé and Shaw, 1989) at chill temperatures. Blickstad *et al.* (1981) compared the microflora on pork stored in atmospheres enriched with CO_2 (Table 5.1). Only one species of Enterobacteriaceae, *Providencia rettgeri*, was found initially and then on only one of the six pork loins tested; it comprised 5% of the microbial flora on that loin. By the end of storage the numbers of Enterobacteriaceae (determined with Violet Red Bile Dextrose medium) had increased by a factor of 10^4 - 10^5 on pork stored in air or in 1 atmosphere of CO_2 at 4 or 14 °C. Much less multiplication had occurred with 5 atm of CO_2. Two species only were detected at the end of storage - *Serratia liquefaciens* and *Enterobacter cloacae* (Table 5.1). The analysis of species was done with isolates taken from plates used for the total aerobic count rather than from the selective medium. In all instances the viable counts on the former were larger than those on the latter.

Table 5.1 Prevalence of Enterobacteriaceae on pork under various storage conditions

Storage conditions		Numbers of micro-organisms at the end of storage (log *n* / cm^2)		Main species of Enterobacteriaceae (% **total** population)
Atmosphere	Temperature (°C)	Total count (TGE[a])	Enterobacteriaceae (VRBD[b])	
Initial[c]	-	3.2	0.5	*Providencia rettgeri* 1%
Air	4	7.0	4.4	*Enterobacter cloacae* 2%
	14	7.8	5.7	*Serratia liquefaciens* 4%
1 atm CO_2	4	7.1	5.5	-
	14	6.3	5.4	*Serratia liquefaciens* 16%
5 atm CO_2	4	7.0	1.1	-
	14	7.0	<4.0	-

Data from Blickstad *et al.* (1981)
a Tryptone glucose extract agar
b Violet red bile dextrose agar
c Initial values taken as an average of six sampled pork loins
\- Enterobacteriaceae were not identified

The incidence of Enterobacteriaceae from the time of slaughter and processing in an abattoir through to meats prepared for retail sale was studied by Stiles and Ng (1981b). *Escherichia coli* and *Serratia liquefaciens* were present at all stages. The latter together with *Pantoea agglomerans* were predominant in ground beef supplied to retail outlets. *Serratia liquefaciens* has been found by many investigators to be the most common

Table 5.2 Enterobacteriaceae isolated from meats

Meat	Conditions of storage			Enterobacteriaceae		Authors[b]
	Temp. (°C)	Atmosphere	Time (d)	%[c]	Species	
Lamb	-1	Low oxygen	42	*	*Serratia liquefaciens*	1
		No oxygen	56	*	*Citrobacter* spp.	
				*	*Enterobacter aerogenes*	
N.S.	Ab[d]	Ab	0	51	*Ent. cloacae*	2
				22	*Klebsiella pneumoniae*	
				15	*Ent. aerogenes*, also *Ent. liquefaciens*, *Serratia* spp.	
Beef (High pH)	0-2	VP	56	93	*Ser. liquefaciens*	3
				7	*Hafnia* spp.	
Pork	14	Air	3	4[e]	*Serratia* spp.	4
		CO_2	6	16	*Serratia* spp.	
	4	Air	8	2	*Ent. cloacae*	
N.S.	Ab	Ab	0	19	*Escherichia coli*	5
				10	*Citrobacter* spp.	
				3	*Ent. aerogenes*	
				9	*Ent agglomerans*	
				9	*Ent. cloacae*	
				4	*Ent. hafniae*	
				22	*Klebsiella* spp.	
				14	*Ser. liquefaciens*	
Sausage	[f]	[f]	[f]	40	*Ent. agglomerans*	6
				16	*Citrobacter freundii*	
				29	*Haf. alvei*	
				15	*Ser. liquefaciens*	
Pork	0-3	VP	14	25[e]	*Ser. liquefaciens*	7
				3	*Ent. agglomerans*	
			70	5	*Ser. liquefaciens*	
				2	*Ent. agglomerans*	
		VP + N_2 flush	14	24	*Ser. liquefaciens*	
			70	6	*Ser. liquefaciens*	
				3	*Ent. agglomerans*	
				11	*Hafnia alvei*	
Beef	4	Barrier bag - no evacuation	3	13[e]	*Ser. liquefaciens*	8
			9	60	*Ser. liquefaciens*	
Beef	6	25% CO_2 + 75% O_2	11[g]	20[e]	Enterobacteriaceae	9
			11[h]	5	Enterobacteriaceae	
Beef	2	50% CO_2 + 15% O_2 + 35% N_2	0	28	*Enterobacter* spp.	10
				48	*Serratia* spp.	
				20	*Hafnia* spp.	
				16	*Yersinia* spp.	
				8	*Citrobacter* spp.	
			28	7	*Enterobacter* spp.	
				52	*Serratia* spp.	
				19	*Hafnia* spp.	
				21	*Yersinia* spp.	

a *Ent. liquefaciens* is synonymous with *Ser. liquefaciens* (see Jones, 1988), *Ent. hafniae* with *Haf. alvei* (see Greipsson and Priest, 1983), and *Ent. agglomerans* with *Pant. agglomerans* (Gavini *et al.*, 1989)

b 1 Newton *et al.* (1977a); 2 Newton *et al.* (1977b); 3 Patterson and Gibbs (1977); 4 Blickstad *et al.* (1981); 5 Stiles and Ng (1981b); 6 Banks and Board (1982); 7 Lee *et al.* (1985); 8 Ahmad and Marchello (1989); 9 Nortjé and Shaw (1989); 10 Manu-Tawiah *et al.* (1991)

c Percentage of species within the Enterobacteriaceae population

* Proportion not specified

N.S. Not specified

d Abattoir study

e Proportion of species within the total microbial population

f Study included meat from fresh and stored (4, 10. 15 or 22 °C for up to 8 days) unsulphited sausages

g Meat aged by hanging for 7days

h Meat aged in vacuum pack for 7 days

member of this family on meat taken from abattoirs or stored in atmospheres of different composition (Table 5.2). Thus, with vacuum packaged (VP) pork stored at 0-3 °C, the proportion of *Ser. liquefaciens* decreased during storage (Lee *et al.*, 1985). *Hafnia alvei*, another common contaminant of meats (Table 5.6), became the dominant member of the Enterobacteriaceae in vacuum packed pork flushed initially with nitrogen (Lee *et al.*, 1985). Relatively high proportions of *Enterobacter cloacae* and *E. aerogenes* were found occasionally (Newton *et al.*, 1977a; Newton *et al.*, 1977b; Blickstad *et al.*, 1981; Stiles and Ng, 1981b; Manu-Tawiah *et al.*, 1991). *Citrobacter* (Newton *et al.*, 1977a; Stiles and Ng, 1981b; Banks and Board, 1983; Manu-Tawiah *et al.*, 1991), *Yersinia* (Manu-Tawiah *et al.*, 1991) and *Klebsiella* (Newton *et al.*, 1977b, Stiles and Ng, 1981b) were minor contaminants only of a range of meats and meat products.

Although members of the family Enterobacteriaceae do not become a numerically dominant part of the microbial association on meats, they may contribute to spoilage. Thus, *Haf. alvei* and *Ser. liquefaciens* produced malodourous diamines - putrescine and cadaverine - in vacuum packed beef. Putrescine levels may be enhanced through ornithine production by arginine-utilising strains of lactic acid bacteria (Dainty *et al.*, 1986). These two species together with *Pantoea agglomerans* produced hydrogen sulphide during the aerobic storage of beef (Dainty *et al.*, 1989). *Serratia liquefaciens* did so in vacuum-packed beef of high pH - 6.6 (Patterson and Gibbs, 1977). Dainty *et al.* (1989) associated an "eggy" odour with the growth of Enterobacteriaceae. This odour (ranging from boiled to rotten egg) was considered to be due to the formation of sulphur compounds, including H_2S. Methanethiol and its derivatives were also found in the headspace of packs of meat inoculated with *Haf. alvei*, *Ser. liquefaciens* and *Pant. agglomerans*. A green discolouration of the meat was associated with the growth of the two first-named organisms. This was probably due to the formation of sulphmyoglobin by combination of H_2S and myoglobin (Nicol *et al.*, 1970). The presence of members of the Enterobacteriaceae, especially *Haf. alvei* and *Ser. liquefaciens*, in large numbers in meats is, therefore, of commercial importance. Thus, the effect of modified atmospheres on the growth/survival of members of this group in the packs of beef steaks included in the present study needed to be determined.

Many of the taxonomic studies of Enterobacteriaceae from meat were done before the publication of 8th edition of Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984). Subsequent to its publication there have been a number of changes in the taxonomy of this family. *Buttiauxella* - type species *Butt. agrestis* - was defined as a new genus by Ferragut *et al.* (1981). Representatives of this genus, which are phenotypically

similar to *Citrobacter* spp., are common in fresh water and soil. The species *Escherichia vulneris* was defined by Brenner *et al.* (1982) and included in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). In 1989, *Enterobacter agglomerans* (synonym *Erwinia herbicola*) was defined as the type species, *Pantoea agglomerans*, of a new genus (Gavini *et al.*, 1989). It has been isolated from seeds, water, humans, animals (e.g. the guts of insects) and particularly from plant surfaces.

In general, members of Enterobacteriaceae associated with meats have been adequately characterised using the test methods developed by Edwards and Ewing (1972), Brenner (1984), Holmes *et al.* (1986) and Holmes and Costas (1992). Recently the identification of Enterobacteriaceae has been aided by the production of a probability matrix based on a maximum of 65 phenotypic tests (Holmes *et al.*, 1986; Holmes and Costas, 1992). The results of these studies were used in the present work to select an appropriate battery of tests for the rapid identification of the strains most commonly (Table 5.2) associated with MAP beef steaks. As there was a need to identify a large number of isolates (>800) a rapid, economic and labour saving method was essential. To achieve this objective, a microtitre plate containing 12 tests (Table 5.3) was adopted for characterisation of isolates tentatively identified with Enterobacteriaceae: Gram negative rods which were oxidase negative and fermented glucose. Validity of the method was checked with type strains. A comparison of type and test strains was made also with a commercial kit (API20E, bio Merieux) to endorse the selection of the adopted method.

Table 5.3 Rapid identification of Enterobacteriaceae with microtitre plates*

Organism	Test[a]											
	1	2	3	4	5	6	7	8	9	10	11	12
Hafnia alvei	+[b]	-	+	-	+	-	-	-	+	+	+	+
Serratia liquefaciens	+	-	d	-	+	-	-	+	+	+	-	+
Enterobacter aerogenes	+	-	+	-	+	-	-	+	+	+	+	+
Enterobacter cloacae	+	+	-	d	+	-	-	-	d	+	+	+
Citrobacter freundii	d	d	-	d	+	+	d	-	+	+	+	+
Escherichia coli	d	d	d	-	d	-	+	-	+	+	+	+
Pantoea agglomerans[c]	-	-	-	-	+	-	-	-	d	+	+	+
Klebsiella pneumoniae.	-	-	+	+	+	-	-	+	+	+	+	+
Buttiauxella agrestis	+	-	-	-	+	-	-	-	d	+	+	+
Providencia alcalifaciens	-	-	-	-	+	-	-	-	d	-	-	+

* Information from Holmes and Costas (1992)

a 1 Ornithine Decarboxylase, 2 Arginine Dihydrolase and 3 Lysine Decarboxylase - all in Møller's Medium; 4 Urease - Christensen's Urea Medium; 5 Citrate - Christensen's Citrate Medium; 6 H_2S Production - Triple Sugar Iron, adapted; 7 Indole Production - from Nutrient Broth; 8 Inositol Fermentation, 9 Glycerol Fermentation, 10 Mannitol Fermentation, 11 Rhamnose Fermentation and 12 Glucose Fermentation - all Hugh and Leifson

b + > 85% - < 15% d 16% - 84%

c Previously *Enterobacter agglomerans* (Gavini *et al.*, 1989)

MATERIALS AND METHODS

Isolation

Members of the family Enterobacteriaceae were enumerated on Violet Red Bile Glucose agar (Oxoid or Lab M) made and used according to the manufacturer's instructions but with incubation at 30 °C. After enumeration, a line was drawn across the bottom of the Petri dish of the lowest countable dilution from each sample. Cells were removed with a sterile toothpick from the five colonies closest to the line and transferred to nutrient agar (NA, Lab M). Pure cultures were obtained from isolates streaked on NA on two occasions. The NA was incubated overnight at 30 °C.

Maintenance

Single colonies were transferred from NA in a Petri dish to a NA slope in a bijou bottle. These were incubated overnight at 30 °C. For short-term storage, the bottles were kept at 4 °C. The method of Rhodes (1957) was used for long-term storage. The entire surface of the NA was immersed in sterile liquid paraffin to prevent dehydration. Again the cultures were stored at 4 °C.

Type strains of *Citrobacter freundii* (NCTC NC09750), *Enterobacter cloacae* (NCTC NC10005), *Hafnia alvei* (NCTC NC08105), *Pantoea agglomerans* (NCTC NC09381) and *Serratia liquefaciens* (NCTC NC10861) were maintained in the same manner and used alongside recently isolated strains to validate the selected methods.

Characterisation

Preliminary characterisation was done with the Gram stain, cell morphology, oxidase test and mode of glucose metabolism [modified Hugh and Leifson medium, Harrigan and McCance (1976)]. Gram negative, oxidase negative rods which fermented glucose were presumed to be Enterobacteriaceae.

Further characterisation was based on a battery of biochemical tests in a microtitre plate method. The media (Table 5.3) were made up in a concentrated (X1.3) form - the equivalent of 100 ml of substrate dissolved in 75 ml of water. With the exception of heat-labile substrates, the media were autoclaved (121 °C for 15 minutes). Stock solutions (10X) of the heat-labile substrates were filter-sterilised and added to the autoclaved basal media. The following media were used (% w/v):

Møller's Medium
adapted from Harrigan and McCance (1976)

Peptone	0.5
Lab lemco	0.5
Glucose	0.05
Pyridoxal	0.005
Phenol red	0.002

pH 6.0

Ornithine, arginine or lysine HCL (10X stock)	1% (L) or 2% (DL)

Citrate
adapted from Difco manual (1984)

Yeast extract	0.05
Cysteine HCl	0.01
$Na_3C_6H_5O_7.3H_2O$	0.3
Glucose	0.02
KH_2PO_4	0.1
NaCl	0.5
Phenol red	0.002

pH Not specified

H_2S production
adapted from Oxoid manual, (1990)

Lab lemco	0.3
Yeast extract	0.3
Peptone	2.0
NaCl	0.5
Glucose	0.1
Ferric citrate	0.03
$Na_2S_2O_3.5H_2O$	0.03

pH 7.4

Indole
Holmes and Costas (1992)

Nutrient broth (pH 7.4) with Kovac's reagent added after incubation.

Urease
Harrigan and McCance (1976)

Peptone	0.1
NaCl	0.5
KH_2PO_4	0.2
Glucose	0.1
Phenol red	0.002

pH 6.8-6.9

Urea	2.0
100X stock f.s.	

Carbohydrate fermentation
Harrigan and McCance, 1976)

Peptone	0.2
NaCl	0.5
K_2HPO_4	0.03
Bromothymol blue	0.003
Carbon sources	0.5 (f.s.)

pH 7.1

Inositol, glycerol, mannitol, rhamnose and glucose (10X stock solutions)

The media were dispensed (150µl per well) into a microtitre plate. Cells from a single colony on nutrient agar were suspended in one ml of sterile water in an Eppendorf tube. Fifty µl of the suspension were used to inoculate each well. The inoculated media for ornithine decarboxylase, arginine dihydrolase, lysine decarboxylase, urease and H_2S tests were overlaid with sterile liquid paraffin. The plates were incubated at 30 °C for up to 48 h with the first examination after overnight incubation. For tests containing phenol red as the pH indicator, a pink colour was scored as positive. Hydrogen sulphide production caused a black precipitate. Indole production was determined following the addition of Kovac's (see

Harrigan and McCance, 1976) reagent (25 µl) to the nutrient broth. A pink colour at the surface of the broth was scored as positive. Carbohydrate fermentation tests were recorded as positive if the colour of broth cultures changed from blue to yellow. A blue colour was scored as negative (Figure 5.1).

A few uncharacteristic profiles were noted during the analysis of the organisms from the Salmonella II trial. Eighteen strains having the profile, + - + - + - - - + + - +, could not be identified. These strains shared certain phenotypic properties with *Enterobacter aerogenes*, *Hafnia alvei* and *Serratia liquefaciens*. Two additional carbon sources, sorbitol and sucrose, were used with these isolates. The fermentation of rhamnose was also repeated to ensure that the initial profile was correct. These tests were done in test tubes containing eight ml of the appropriate carbohydrate fermentation medium (pp122). The results were recorded after overnight incubation at 30 °C. Table 5.4 shows the profiles expected for the species having characteristics most similar to the original unidentified profile.

Table 5.4 Differentiation of three phenotypically related Enterobacteriaceae*

	Additional tests adopted for strains with uncharacteristic profiles		
	Rhamnose	Sorbitol	Sucrose
Enterobacter aerogenes	+[a]	+	+
Hafnia alvei	+	d	-
Serratia liquefaciens	-	+	+

* Information from Holmes and Costas (1992)

a + > 85% - < 15% d 16% - 84%

API 20E Strips (bio Merieux)

API 20E strips were used according to the manufacturer's instructions to validate the method described above. Isolates were grown overnight on NA at 30 °C. A single colony was suspended in 5 ml of sterile distilled water using a wooden applicator. Each well of the strip was filled to the appropriate level with a cell suspension and, where specified, wells were overlaid with sterile liquid paraffin. Strips were incubated at 30 °C and read after 18 - 24 h.

Reproducibility

Ten % of the isolates were re-tested to determine the reproducibility of the method. The results were analysed using the following equation:

$$\frac{\text{Number of tests giving the same reaction}}{\text{Total number of tests}} \times 100$$

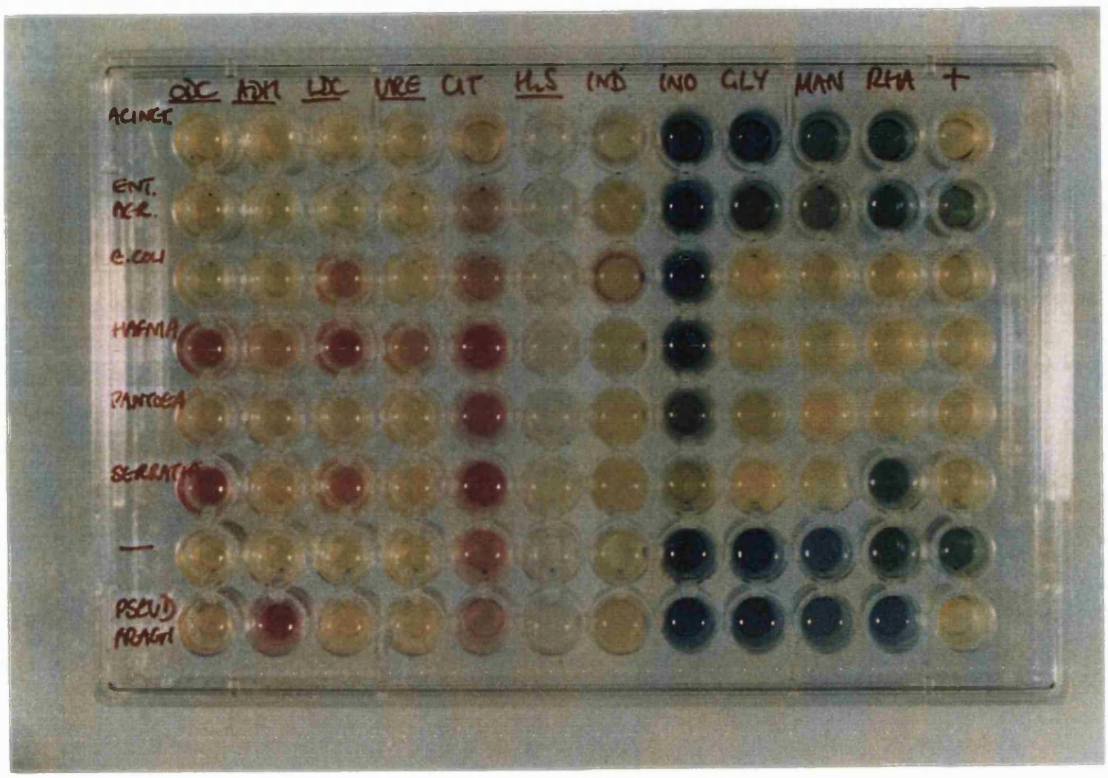


Figure 5.1 **Characterisation of members of the family Enterobacteriaceae using a microtitre plate method.**

Acineto., *Acinetobacter* sp.; Ent. aer, *Enterobacter aerogenes* (culture contaminated therefore not showing expected results); E. coli, *Escherichia coli*; Hafnia, *Hafnia alvei*; Pantoea, *Pantoea agglomerans*; Serratia, *Serratia liquefaciens*; -, No culture inoculated; Pseud. fragi *Pseudomonas fragi*

ODC, Ornithine decarboxylase; ADH, Arginine dihydrolase; LDC, Lysine decarboxylase; URE Urease; CIT, Citrate; H_2S, Hydrogen sulphide; IND, Indole; Fermentation of: INO, Inositol; GLY, Glycerol; MAN, Mannitol; RHA, Rhamnose; +, Glucose

RESULTS

A total of 90 strains were used to ascertain the accuracy of the microtitre plate method *vis à vis* API strips (Tables 5.5). The microtitre plate method gave results equivalent to, or better than, those from API strips. As fewer unidentified isolates or those giving dubious profiles were obtained with the microtitre plate, it was used in further studies.

Having established the validity of the microtitre plate method, it was used to characterise over 600 isolates from beef steaks packed in modified atmospheres. Overall, *Hafnia alvei* was the most commonly isolated species (Table 5.6). There was variation between the results obtained at the end of storage of the different trials, *viz.* This species was >40% of the enterobacteriaceae on meat stored at both temperatures in the first two trials and 8% in Salmonella II trial. The total percentages (Table 5.6) showed that *Haf. alvei, Serratia liquefaciens* and *Pantoea agglomerans* were the most common members of the enterobacteriaceae at the end of storage. The other species were minor contaminants only.

Table 5.5 Evaluation of the accuracy of the microtitre plate method for the identification of members of the family Enterobacteriaceae

	Numbers identified by:	
Organism identified	API	Microtitre plate
Pantoea agglomerans	13	16
Serratia liquefaciens	12	13
Citrobacter freundii	1	2
Providencia alcalifaciens	3	4
Escherichia/Shigella	1	0
Hafnia alvei	43	46
Enterobacter aerogenes	4	5
Unidentifed/doubtful profile	13	4

The selectivity of violet red bile glucose agar (VRBG) proved to be unsatisfactory; contamination of the medium by pseudomonads was a common feature. Over 800 strains from VRBG were isolated and purified. Of these, only 606 were assigned to Enterobacteriaceae and characterised further. The growth of pseudomonads was due, in part, to incubation at 30 °C. The recommended temperature for VRBG is 37 °C, but psychrotrophic strains of Enterobacteriaceae on meat stored at chill temperatures may be unduly stressed by the selective medium at 37 °C. Sometimes colony size allowed presumptive identification of pseudomonads - these organisms formed relatively small colonies *vis à vis* those of Enterobacteriaceae. There were occasions, however, when the colonies of both groups were of similar but intermediate size.

Table 5.6 Prevalence of members of Enterobacteriaceae on beef steaks at the end of storage in modified atmospheres stored at chill temperatures

	% of VRBG[a] isolates					
	Listeria		Salmonella I[b]		Salmonella II	Total
Organism identified	0 °C	5 °C	0 °C	5 °C	5 °C	
Pantoea agglomerans	8		60	5	33	21
Serratia liquefaciens	28	14		19	39	28
Enterobacter aerogenes	4	5			12	6
Enterobacter cloacae					2	<1
Escherichia/Shigella					4	2
Escherichia vulneris					1	<1
Hafnia alvei	53	82	40	77	8	37
Providencia alcalifaciens	6					2
Klebsiella pneumoniae					<1	<1
Citrobacter freundii	1					<1
Buttiaxella agrestis					1	<1

a Violet red bile glucose agar
b Very few isolates were Enterobacteriaceae, most were pseudomonads isolated from VRBG

Having demonstrated the overall importance of *Haf. alvei, Ser. liquefaciens* and *Pant. agglomerans* in modified atmosphere packaged beef steaks stored at chill temperatures, the effects of the storage conditions were assessed. This was done in three separate studies, each of which involved beef steaks packed under vacuum (VP), or in 50% N_2 + 50% CO_2 (50/50), 80% O_2 + 20% CO_2 (80/20) or 100% CO_2 (CO_2). Packs were stored at 0 or 5 °C and isolates purified from both temperatures in two of the trials. In the final trial at 5 or 12 °C, only organisms present at the lower temperature were isolated and identified.

In the first trial - with meat deliberately inoculated with *Listeria monocytogenes* - two packs at each temperature and atmosphere were sampled. Each pack was quartered and two samples taken. Thus there were 20 isolates for each atmosphere at both temperatures. The variation of species within and between the packs is shown (Table 5.7). There was little difference between or within packs and subsequent studies used a single sample from each of two packs. Thus ten isolates for each atmosphere and temperature at every sampling time were identified.

Lactic acid bacteria were found to dominate the microflora on MAP beef steaks in all atmospheres tested. The size of the population of Enterobacteriaceae remained static or diminished slightly during storage at 0 °C (see Chapter 2, pp.). The exception was the high oxygen (80%) atmosphere, where, after an initial increase, the numbers of Enterobacteriaceae diminished (one log cycle below that of the initial population). At 5 °C their numbers remained unchanged in an atmosphere containing carbon dioxide alone, increased slightly in vacuum packs and in 50/50, but extensively (to over $10^7 g^{-1}$) in 80/20 by the end of storage.

Table 5.7 Study of the variation of Enterobacteriaceae within and between packs at the end of storage for the Listeria trial

a) Uninoculated 0 °C

	Vacuum Pack				50% O_2 + 50% CO_2				80% O_2 + 20% CO_2				100% CO_2			
Samples:[a]	1		2		1		2		1		2		1		2	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Day of sample	70	70	70	70	70	70	70	56	56	56	31	31	31	31	56	56
Hafnia[b]	**5**	**5**	**4**	**4**	**2**			**1**				**2**				
Serratia			**1**	**1**	**2**		**4**	**3**	**4**							
Pantoea						**5**	**1**	**1**								
Enterobacter																
Citrobacter																
Providencia																**2**
Pseudomonas					**1**				**1**	**5**	**5**	**3**	**5**	**5**	**5**	**3**

b) Inoculated 0 °C

	Vacuum Pack				50% O_2 + 50% CO_2				80% O_2 + 20% CO_2				100% CO_2			
Samples:[a]	1		2		1		2		1		2		1		2	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Day of sample	77	77	77	63	77	63	77	77	63	63	63	49	49	49	49	49
Hafnia[b]	**4**	**5**	**5**	**5**	**3**	**4**	**4**	**3**		**1**						
Serratia	**1**				**2**	**1**	**1**	**1**	**3**	**1**	**4**	**1**				
Pantoea										**1**		**1**				
Enterobacter								**1**	**2**		**1**					
Citrobacter													**1**			
Providencia													**1**	**2**	**2**	
Pseudomonas										**2**		**3**	**3**	**3**	**3**	**2**[c]

a 1 and 2 correspond to duplicate packs, A and B correspond to duplicate quarters within a pack

b *Hafnia alvei, Serratia liquefaciens, Pantoea agglomerans, Enterobacter aerogenes, Citrobacter freundii, Providencia alcalifaciens, Pseudomonas* spp.

c Only 2 strains grew on sub-culture

Table 5.7 contd. **Study of the variation of Enterobactereaceae within and between packs at the end of storage for the Listeria trial**

c) Uninoculated 5 °C

	Vacuum Pack				50% O_2 + 50% CO_2				80% O_2 + 20% CO_2				100% CO_2			
Samples:[a]	1		2		1		2		1		2		1		2	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Day of sample	34	34	34	34	29	24	24	24	29	24	29	29	34	34	34	34
Hafnia[b]	**5**	**5**	**5**	**5**	**5**	**5**	**4**	**4**	**1**			**2**	**5**	**5**	**5**	**5**
Serratia							**1**	**1**	**3**	**1**	**3**	**2**				
Pantoea																
Enterobacter									**1**		**2**	**1**				
Citrobacter																
Providencia																
Pseudomonas										**4**						

d) Inoculated 5 °C

	Vacuum Pack				50% O_2 + 50% CO_2				80% O_2 + 20% CO_2				100% CO_2			
Samples:[a]	1		2		1		2		1		2		1		2	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Day of sample	NP	34	34	34	29	29	29	29	24	24	24	24	34	34	34	34
Hafnia[b]		**5**	**5**	**4**	**5**	**4**	**3**	**2**		**1**	**1**		**4**	**4**	**3**	**5**
Serratia						**1**	**2**	**2**		**1**			**1**		**1**	
Pantoea																
Enterobacter								**1**						**1**		
Citrobacter																
Providencia																
Pseudomonas				**1**					**5**	**3**	**4**	**5**			**1**	

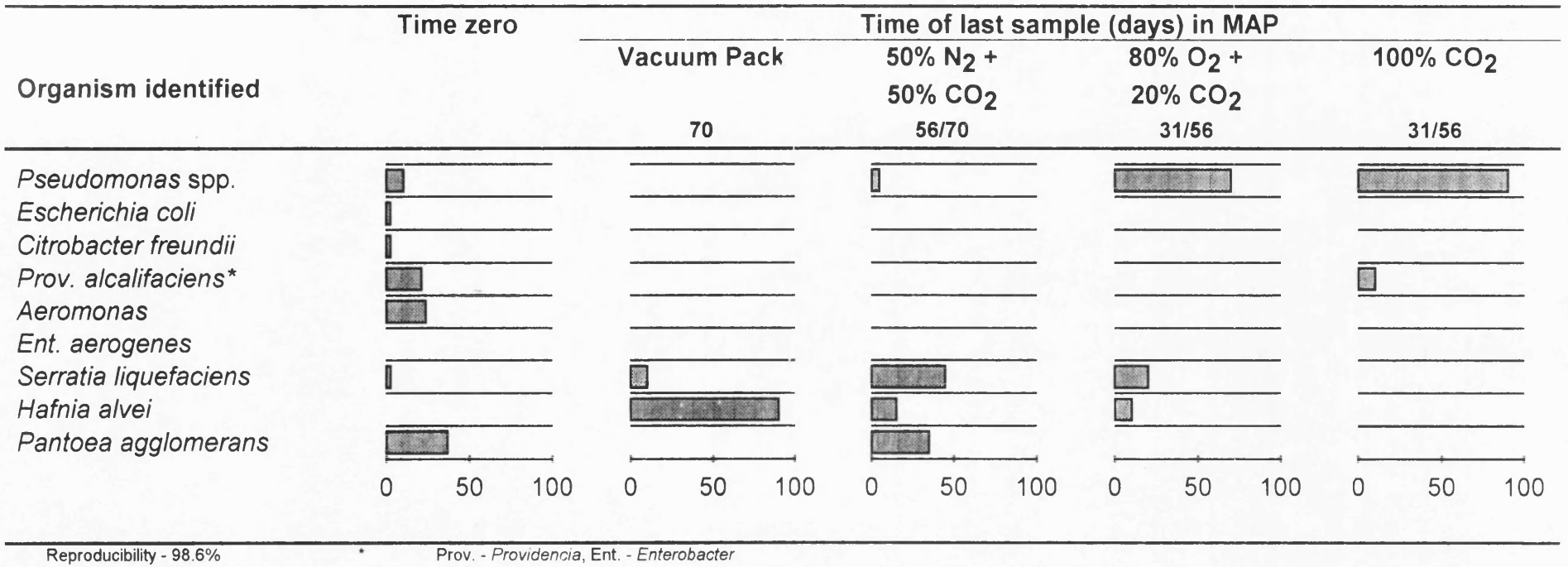
a 1 and 2 correspond to duplicate packs, A and B correspond to duplicate quarters within a pack

b *Hafnia alvei, Serratia liquefaciens, Pantoea agglomerans, Enterobacter aerogenes, Citrobacter freundii, Providencia alcalifaciens, Pseudomonas* spp.

Beef steaks in the Listeria trial harboured initially a consortium of Enterobacteriaceae species (Figure 5.2). *Pantoea agglomerans* was the most common isolate and *Providencia alcalifaciens* also occurred at a relatively high incidence. *Escherichia coli, Citrobacter freundii* and *Serratia liquefaciens* made small contributions to the population. Some of the VRBG isolates were assigned to the genera *Pseudomonas* or *Aeromonas.* These were not characterised further. By the end of storage at 0 °C in uninoculated (control) packs, *Hafnia alvei,* which was not present initially, was dominant in VP and present in small numbers in 50/50 and 80/20 atmospheres. *Serratia liquefaciens* was also present on meat stored in these atmospheres. Pseudomonads were the major isolates from the atmosphere containing CO_2 alone, the only Enterobacteriaceae isolate being *Prov. alcalifaciens. Pantoea agglomerans* persisted to the end of storage in one atmosphere only, namely 50/50. Similar trends were noted in packs inoculated with *Listeria monocytogenes* (Figure 5.2b). The meat stored in the 50/50 atmosphere did not contain *Pantoea* (present only in the 80/20 packs). A few *Enterobacter aerogenes* were isolated and *Haf. alvei* was present in relatively high numbers. *Enterobacter aerogenes* was also isolated from meat stored in 50/50 and 80/20 atmospheres. *Citrobacter freundii* formed a very small part of the microflora on meat stored in CO_2.

Different trends in the analysis of meat stored at 5 °C were noted (Figure 5.3). *Hafnia alvei* was dominant in all atmospheres other than 80/20 in which it was a minor part of the microbial consortium. Meat in an 80/20 atmosphere contained the most diverse flora with *Ser. liquefaciens* > *Ent. aerogenes* > *Haf. alvei* and a few *Aeromonas* spp. present. Packs inoculated with *Listeria monocytogenes* tended to have common patterns of contamination with various Enterobacteriaceae but pseudomonads comprised the largest proportion in the 80/20 atmosphere.

The second and third trials were concerned with meat inoculated with *Salmonella typhimurium*; the second trial (Salmonella I trial) involved beef steaks stored at 0 or 5 °C, whilst the third trial (Salmonella II) compared storage at 5 with the severe abuse temperature of 12 °C. *Salmonella typhimurium,* being a member of the Enterobacteriaceae, grew on the selective medium, VRBG. In the second trial very few uninoculated packs were included and this resulted in only 58 strains of Enterobacteriaceae species being identified. The initial microflora isolated on VRBG was exclusively pseudomonads. These organisms persisted throughout storage. Of the 58 Enterobacteriaceae, 15 were from beef steaks stored at 0 °C; of these 60% were *Pant. agglomerans* and 40% *Haf. alvei.* Of the remainder

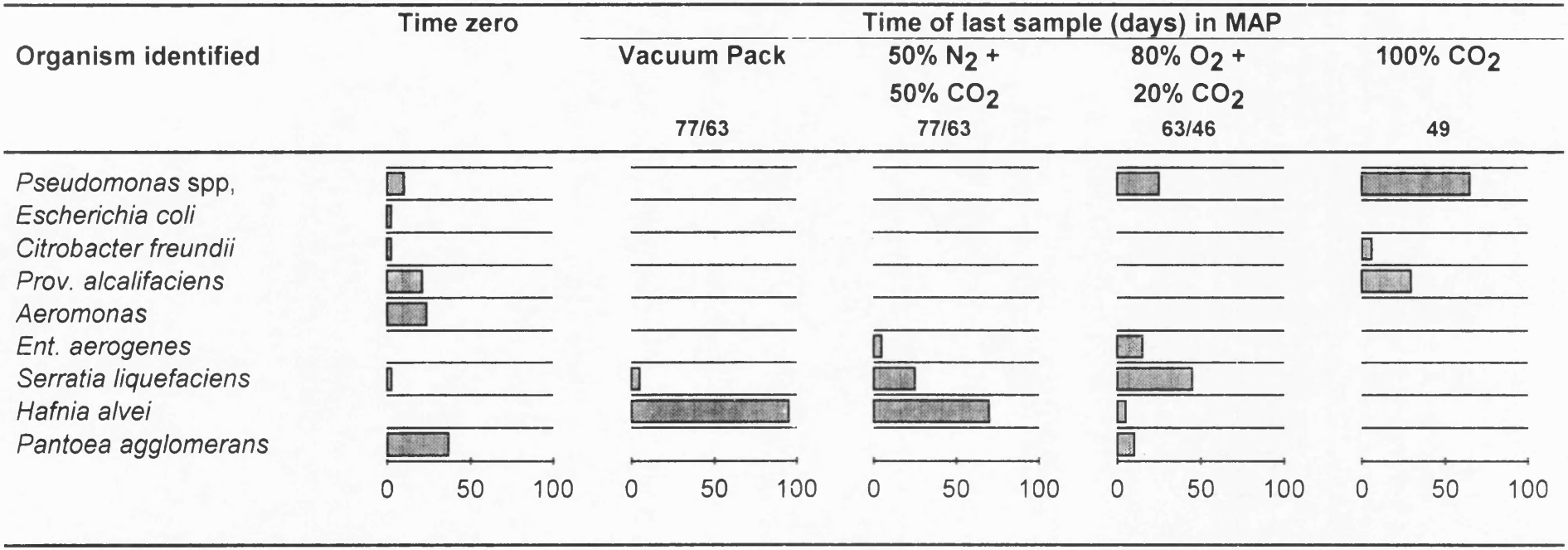


Reproducibility - 98.6%

* Prov. - *Providencia*, Ent. - *Enterobacter*

a) Uninoculated packs

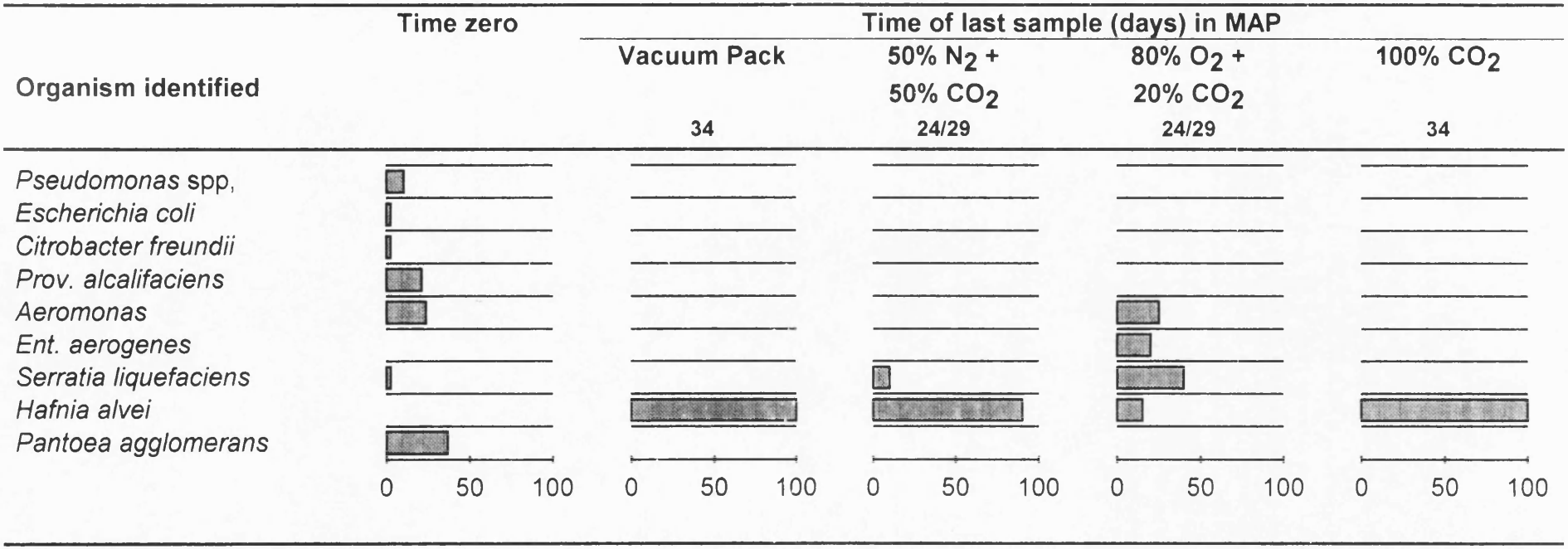
Figure 5.2 Changes in the population of Enterobacteriaceae from modified atmosphere packaged beef steaks stored at 0 °C - Listeria trial



Reproducibility - 98.6%

b) Inoculated packs

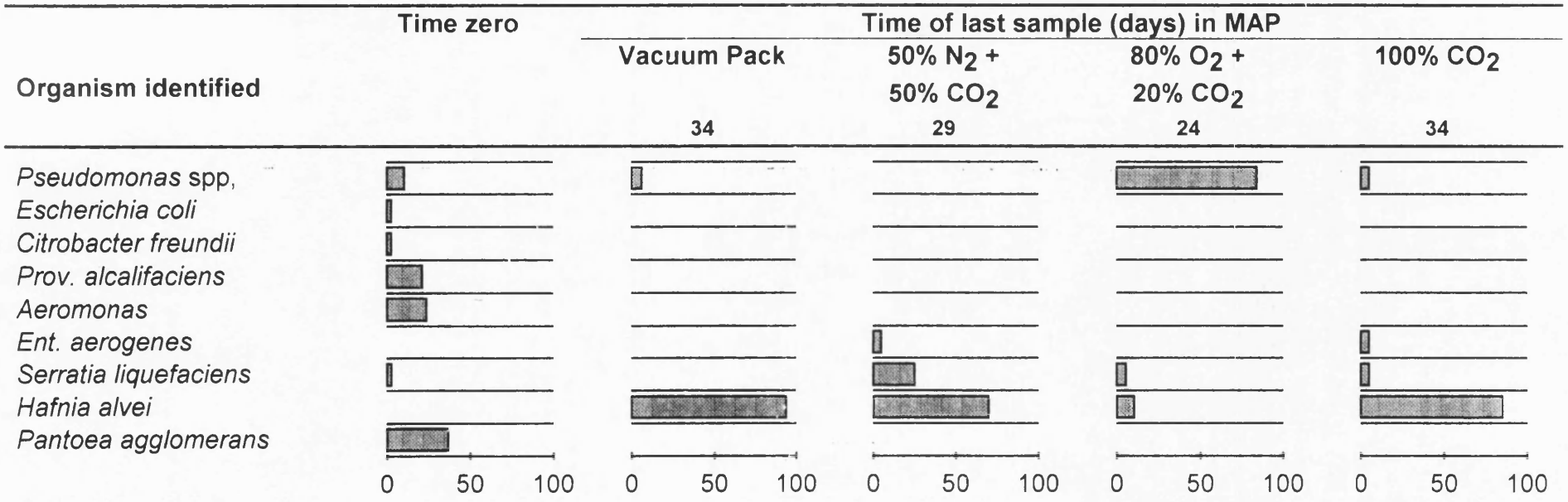
Figure 5.2 contd. **Changes in the population of Enterobacteriaceae from modified atmosphere packaged beef steaks stored at 0 °C - Listeria trial**



Reproducibility - 98.6%

a) **Uninoculated packs**

Figure 5.3 contd. **Changes in the population of Enterobacteriaceae from modified atmosphere packaged beef steaks stored at 5 °C - Listeria trial**



Reproducibility - 98.6%

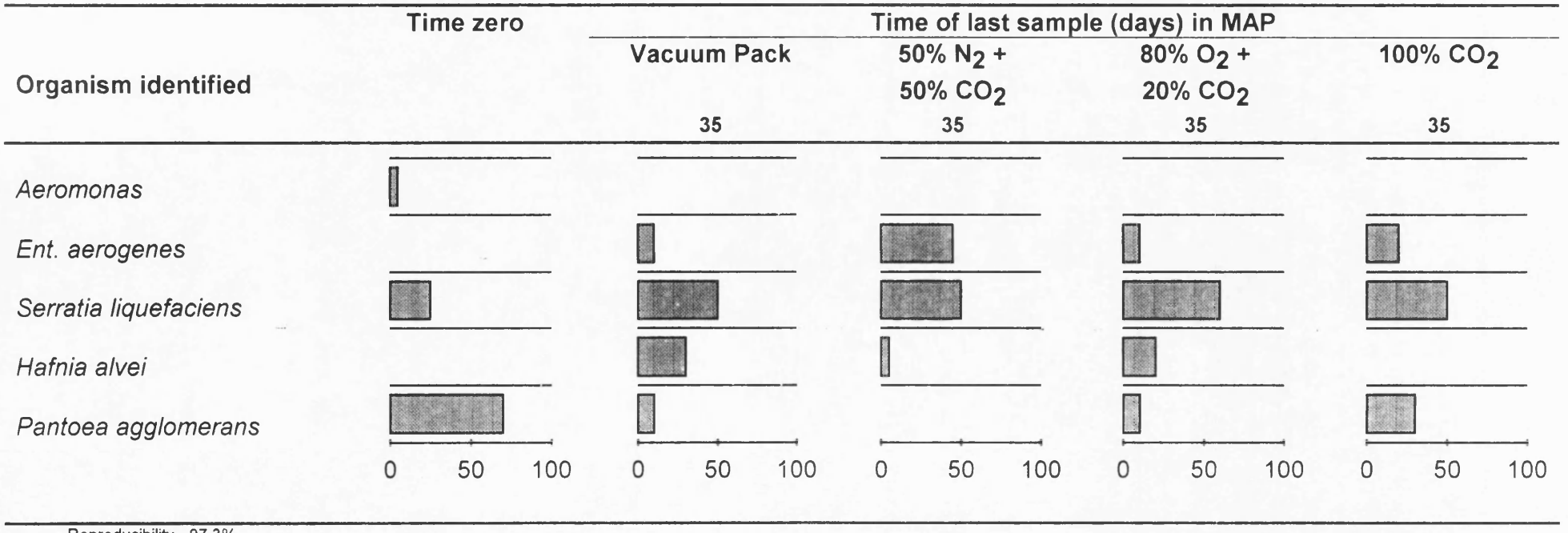
b) Inoculated packs

Figure 5.3 contd. **Changes in the population of Enterobacteriaceae from modified atmosphere packaged beef steaks stored at 5 °C - Listeria trial**

of the isolates from meat stored at 5 °C, the majority (77%) were identified with the latter species. *Serratia liquefaciens* comprised 19% and *Pantoea agglomerans* 5% of the Enterobacteriaceae isolates. No attempt was made to compare the microflora present in the different atmospheres because of the small numbers of isolates.

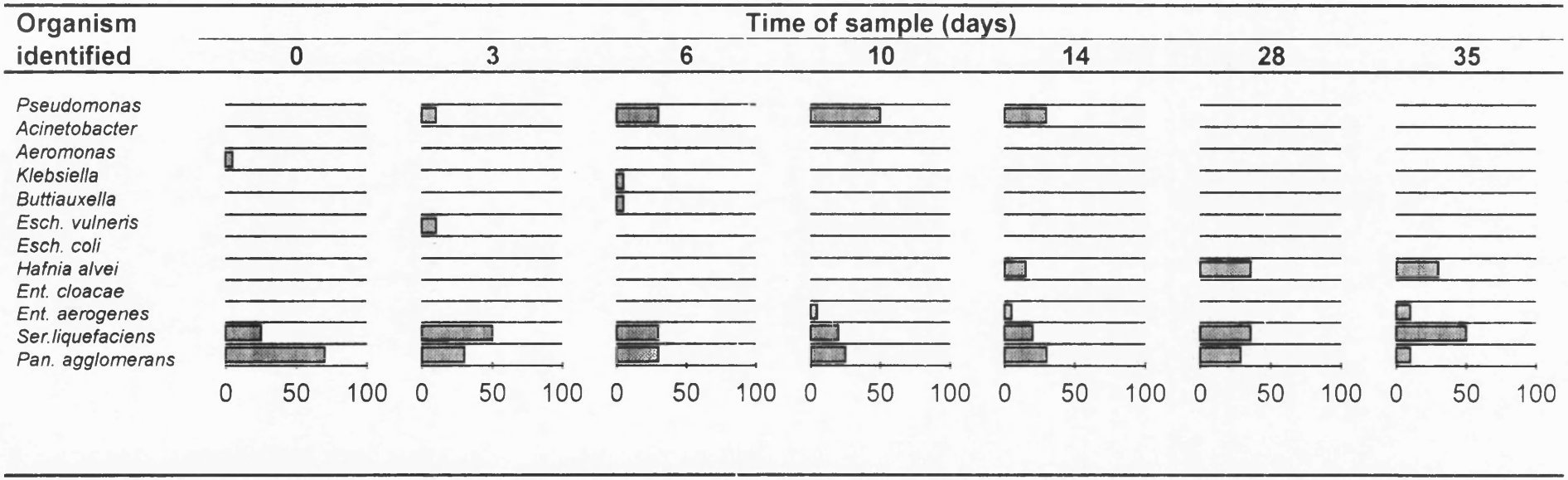
The second Salmonella trial included a large number of control (uninoculated) packs. These samples were analysed throughout storage but the results only of those at the beginning and end of the storage period are presented in Figure 5.4. At the time of packing *Pantoea agglomerans* was the dominant member of the Enterobacteriaceae - as it was in the initial (Listeria) trial. It comprised 70% of the initial population (*cf.* 36.8% in the Listeria trial) and persisted to the end of storage in all atmospheres except 50/50. In comparison with the Listeria trial, *Ser. liquefaciens* was also present in large numbers initially and it was the dominant member of the Enterobacteriaceae in all atmospheres by the end of storage. *Hafnia alvei*, which was dominant in the Listeria trial, occurred in relatively small numbers throughout storage (Figure 5.3). It was completely inhibited in the atmosphere containing only CO_2. *Enterobacter aerogenes*, which was present on meats at the end of storage in all atmospheres, comprised about 50% of the Enterobacteriaceae on meat in the 50/50 atmosphere.

The beef steaks in this trial (Salmonella II trial) were analysed throughout storage such that changes within the population of Enterobacteriaceae were determined (Figure 5.5). The numbers of *Pantoea agglomerans*, the dominant species at the outset of the trial, tended to diminish throughout storage whereas those of *Ser. liquefaciens* remained unchanged or increased. From day 14 onwards *Hafnia alvei* was isolated from meat in VP and 50/50 (Figure 5.5a and b), but was present only on days 10 and 35 in 80/20 and day 28 in CO_2. At all times it comprised less than 25% of the isolates from VRBG. A similar trend was noted with *Ent. aerogenes* - it was present only in the later stages of storage. *Klebsiella pneumoniae*, *Buttiauxella agrestis*, *Escherichia vulneris* and *Esch. coli* were isolated occasionally.



Reproducibility - 97.3%

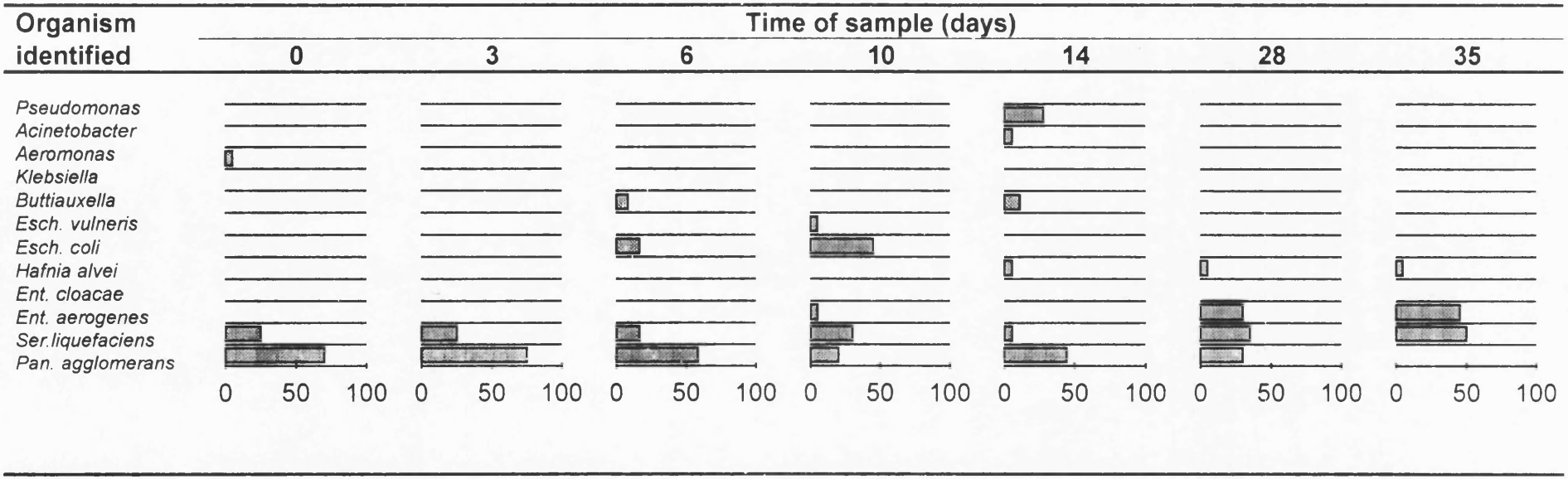
Figure 5.4 **Changes in the population of Enterobacteriaceae from modified atmosphere packaged beef steaks stored at 5 °C - Salmonella II trial**



Reproducibility - 97.3%

a) Vacuum packs

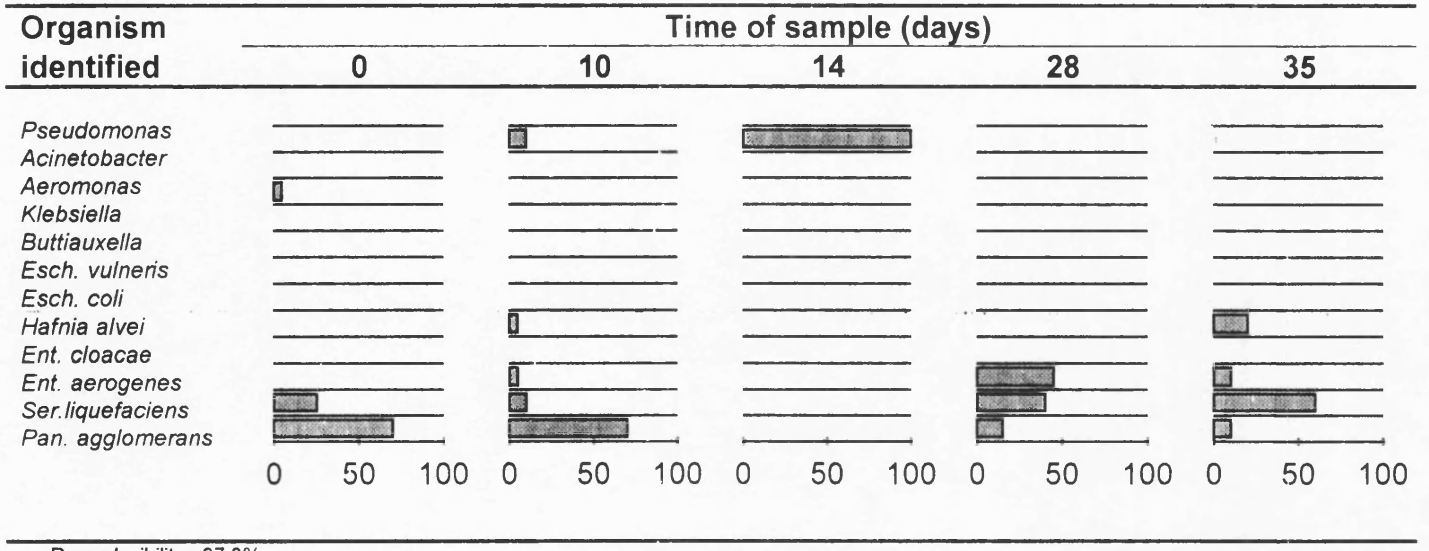
Figure 5.5 **Changes in the population of Enterobacteriaceae on modified atmosphere packaged beef steaks stored at 5 °C - Salmonella II trial**



Reproducibility - 97.3%

b) 50% N_2 + 50% CO_2

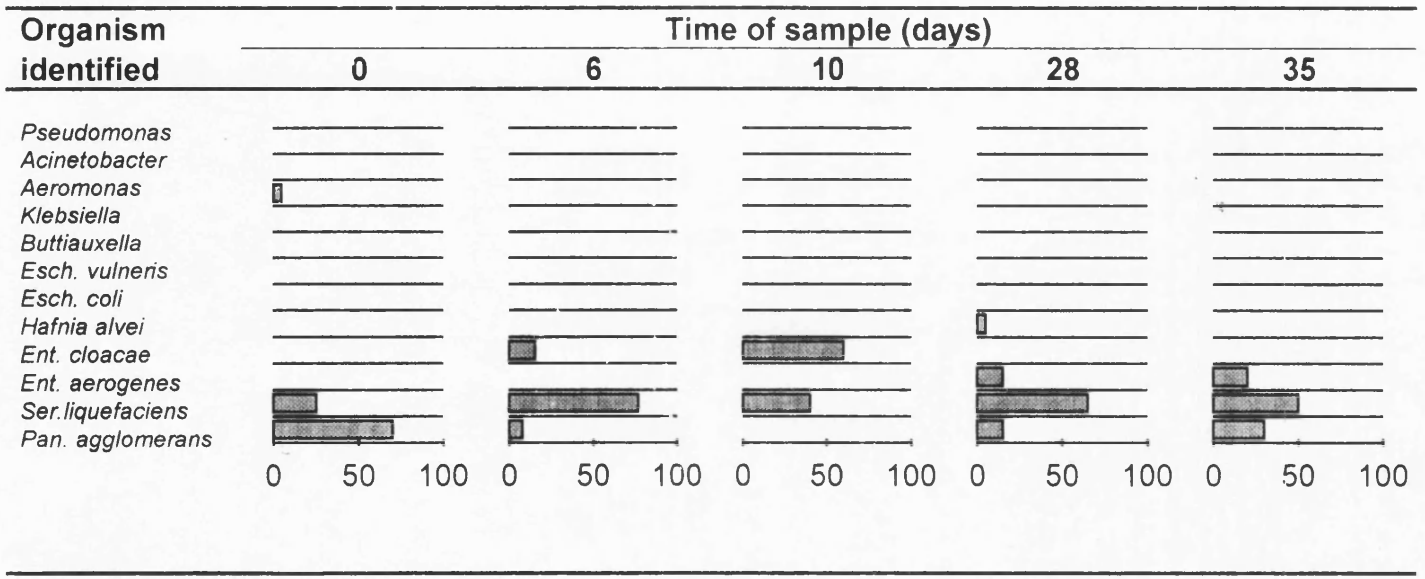
Figure 5.5 contd. **Changes in the population of Enterobacteriaceae from modified atmosphere packaged beef steaks stored at 5 °C - Salmonella II trial**



Reproducibility - 97.3%

c) 80% O_2 + 20% CO_2

Figure 5.5 contd. **Changes in the population of Enterobacteriaceae from modified atmosphere packaged beef steaks stored at 5 °C - Salmonella II trial**



Reproducibility - 97.3%

d) 100% CO_2

Figure 5.5 contd. **Changes in the population of Enterobacteriaceae from modified atmosphere packaged beef steaks stored at 5 °C - Salmonella II trial**

DISCUSSION

The comparison of the microtitre plate method with the commercial kit (API20E, bio Merieux) demonstrated that the method devised for the identification of Enterobacteriaceae in this study was soundly based. The API20E kit (comprising 21 tests with results and identification within 24 h) was tested against the probability matrix of Holmes *et al.* (1986). The identification of a selection of Enterobacteriaceae gave 88% correct identification, 10% of strains were unidentified and 2% were mis-identified (Holmes and Costas, 1992). This compares favourably with other commercial kits. Of the strains isolated from meat in many studies, the majority were readily identified (Holmes *et al.*, 1986). *Enterobacter cloacae* and *Escherichia coli* gave variable results. Stiles and Ng (1981a) found it difficult to distinguish between *Serratia liquefaciens*, *Ent. cloacae* and *Ent. hafniae* (now *Hafnia alvei*). By the adoption of appropriate differential tests the microtitre plate method devised in the present study distinguished between these species. In cases of dubiety due to "doubtful" profiles additional tests were used. These provided the essential information for the identification of such isolates. The methods were adopted for the characterisation and identification of the large numbers of isolates that needed to be examined in order to analyse the effects of storage conditions on the growth/survival of members of the family Enterobacteriaceae.

Violet red bile glucose agar (VRBG), a derivative of violet red bile agar with lactose which is used in the clinical laboratory, was developed for the enumeration of Enterobacteriaceae from foods (see Anon., 1987b). Dark purple colonies (1-2 mm in diameter) surrounded by a purple halo should be included in the count. Ng and Stiles (1978) used VRBG in a study of over 300 meat samples. A differential count of bile-precipitating (purple halo - B+) and non bile-precipitating (no halo - B-) colonies was made. The difference between the total count and that of the bile precipitating colonies was $\log_{10}$ 2.52 where the total count was log 3.076. In other words, the non-bile precipitating colonies comprised 28% of the flora on VRBG. Strains of both colony types were purified and identified. Those from the B+ colonies were mainly *Escherichia coli*, *Ser. liquefaciens* and *Enterobacter* (now *Pantoea*) *agglomerans*. The last named, however, was also the most common member of the B- isolates, comprising 45% of the that colony type. A further 36% of the organisms were oxidase positive. The present study is not directly comparable as a lower incubation temperature was used, 30 *vis à vis* 37 °C in the study of Ng and Stiles (1978). It is obvious, however, that contaminating micro-organisms (commonly

pseudomonads or aeromonads) grow on VRBG. Enumeration of bile-precipitating colonies only is evidently not satisfactory as it does not distinguish Enterobacteriaceae from other micro-organisms.

The occurrence of *Pant. agglomerans, Ser. liquefaciens, Haf. alvei* and *Ent. aerogenes* in meats has been noted previously (Table 5.1). The results of the present study regarding the overall proportions of members of Enterobacteriaceae were in accord with many previous studies also. Generally, Enterobacteriaceae are inhibited in modified atmospheres containing high levels of CO_2 - pCO_2 approaching 1 atm (Gill and Molin, 1991), especially at low temperatures (Egan, 1984). Consequently they do not contribute significantly to the final microbial association (Blickstad and Molin, 1983; Gill and Penney, 1986) except on meat of high pH (Gill and Penney, 1985; Gill and Penney, 1986) or on lamb where they have been found to comprise up to 10% of the microflora (R.H. Madden, pers. comm). The spoilage potential of these organisms is of commercial concern. Indeed, in VP lamb which exhibited an unpleasant odour, *Ser. liquefaciens* and *Ent. aerogenes* were found to be the dominant Gram negative organisms. Strains of these species exhibited an unusually high tolerance for CO_2 (R.H. Madden, pers. comm.). It would be advantageous therefore to inhibit the activity of those species of greatest spoilage potential. Work to date has not demonstrated the effects of particular storage conditions on the development of such species (*Pant. agglomerans, Ser. liquefaciens, Haf. alvei* and *Ent. aerogenes*) during storage in MAP.

The effect of CO_2 was most pronounced on the development of *Haf. alvei.* This gas was less effective at 5 than at 0 °C. In an atmosphere containing CO_2 alone, this organism was not detected at the end of storage of meat at the lower temperature whereas it formed > 80% of the Enterobacteriaceae population in uninoculated and inoculated steaks at 5 °C. Other species did not show significant differences with atmospheres or temperature. Despite the large numbers of organisms identified, the effects of individual storage conditions in particular could not be demonstrated. Factors other than atmosphere and temperature probably have a greater importance in the growth/survival of individual species.

Lactic acid bacteria commonly dominate the microflora of MAP meat. As noted previously the metabolic activity of these organisms produces several antimicrobial agents. Organic acids, including lactic acid (van Netten and Mossel, 1980; Gill and Newton, 1982; Smulders *et al.*, 1986) and particularly acetic acid (Gill and Newton, 1982; Bell *et al.*, 1986), are likely to be relatively effective against Enterobacteriaceae.

CHAPTER 6

PSEUDOMONAS SPP. IN THE MICROBIAL FLORA ON MODIFIED ATMOSPHERE PACKAGED BEEF STEAKS

INTRODUCTION

Early studies (Table 6.1) of the microbiology of aerobically chill-stored meats identified the dominant spoilage organisms with two genera. The non-pigmented, Gram negative motile bacteria (present in highest numbers) were assigned to *Achromobacter* and pigmented ones to *Pseudomonas* (Haines, 1933b; Empey and Scott, 1939). On the basis of motility (polar flagella) and oxidative metabolism of glucose, Brown and Weidermann (1958) later re-identified many strains of *Achromobacter* - isolated from meat by Empey and Scott (1939) - with *Pseudomonas*. Routine methods for the tentative identification of aerobic Gram negative bacteria from chilled foods were developed by Shewan *et al.* (1960) and Thornley (1960). From their work on fish, Shewan *et al.* (1960) proposed a determinative key based on seven tests (motility, morphology of the cell and colony, penicillin sensitivity, reaction in Hugh and Leifon's medium, oxidase test and growth at 37 °C). Thornley (1960) developed the arginine test (anaerobic growth with ammonia production) for the differentiation of *Pseudomonas* (positive) from *Achromobacter* (negative) obtained from chicken stored at chill temperature. The latter genus was found subsequently to be a very heterogeneous collection of organisms and *Achromobacter* was recommended to be *nomen dubium* (Hendrie, Holding and Shewan, 1974). Although these studies helped in the day-to-day work on the microbiology of proteinaceous foods, they underlined the need for a systematic study of *Pseudomonas* as emphasised by Ayres (1960) in his review of meat microbiology. This was done by Stanier *et al.* (1966) in a study which sought to group pseudomonads on the basis of their nutritional versatility. Two hundred and 67 strains from the University of California Culture Collection and other sources were characterised using over 160 tests most of which were concerned with substrate utilisation. Unfortunately, a strain of *Ps. fragi*, a species found to be present in many dairy products (Hussong *et al.*, 1937) and in meats (Ayres, 1960), was not included.

When attempting to identify the important aerobic members of the microbial associations on meats, Davidson *et al.* (1973) followed the methodologies developed by Stanier and his collaborators (1966). Only a minority (16/100) of the pseudomonad strains were identified with those defined by Stanier *et al.* (1966), the majority being motile but non-pigmented and non-proteolytic. Other workers have also noted the dominance of such organisms on chilled proteinaceous foods (Table 6.2).

Table 6.1 **Chronology of the characterisation of pseudomonads from chilled foods**

Author	Year	No. of tests	Test type[a]	No. of strains	Origin
Haines	1933b	N.S.	M/B	120	Meat
Hussong *et al.*	1937	>30	M/B	N.S.	Dairy
Empey and Scott[b]	1939	N.S.	N.S.	N.S.	Beef
Brown and Weidermann	1958	16	M/B	189	CC[c] Beef
Shewan *et al.*	1960	11	M/B	N.S.	
Ayres	1960				
Thornley	1960	9	M/B	141	Chicken
Stanier *et al.*	1966	>168	M/B/G+C	267	CC, Various
Palleroni and Douderoff	1972		D		
Palleroni *et al.*	1972		D		
Davidson *et al.*	1973	150	M/B	100	Beef, pork, lamb, sausage
Byng *et al.*	1980		B	90	
Molin and Ternström	1982	174	M/B	200	meat
Shaw and Latty	1982	168	M/B	110	Spoiled beef and pork
Banks and Board	1983	148	M/B	165	Pork, sausage
de Vos and de Ley	1983		R-D	65	
Shaw and Latty	1984	18	B	787	Beef, pork and lamb
Molin and Ternström	1986	215	M/B	200	Beef, pork
Barrett *et al.*	1986	147	M/B/G+C	170	
Gennari and Dragotto	1992	12	B	119	Beef, pork, lamb, rabbit
Prieto *et al.*	1992	63	M/B	268	poultry, Lamb

a M, Morphological; B Biochemical; G+C mol %; D, DNA homology; R-D, RNA-DNA homology
b Identfication to genus level (*Achromobacter*) only
c CC, Culture collection
N.S. Not specified

A detailed study of meats stored aerobically at 0, 5 or 10 °C and sampled throughout storage (Shaw and Latty, 1984) identified the majority (78%) of the numerically dominant organisms with two biovars of *Ps. fragi*. Less than 5% were identified with two biovars of *Ps. fluorescens* and *Ps. putida* respectively. These findings are in accord with many other studies (see Table 6.2). Eleven % of the isolates of Shaw and Latty (1984) grouped in an un-named cluster possibly related to cluster III of Shaw and Latty (1982) and an un-named group in the study by Molin and Ternström (1982). A group of organisms with similar characteristics was isolated by Molin and Ternström (1986) from beef and pork.

Subsequently these strains were defined as a new species, *Ps. lundensis* (Molin *et al.* 1986). Thus today the meat pseudomonads can be adequately characterised with appropriate tests. This is not the case for the "genus" as a whole. It has been stated recently that "the genus should be redefined and restricted" (Willems *et al.*, 1992). Extension of the work begun by Stanier *et al.* (1966) demonstrated that *Pseudomonas* was composed of at least four distantly related genotypic groups (Palleroni and Douderoff, 1972; Palleroni *et al.*, 1972; Palleroni *et al.*, 1973; Byng *et al.*, 1980; de Vos and de Ley, 1983). Indeed the genus *Pseudomonas* was separated (Palleroni, 1984) into four rRNA groups in the latest edition of *Bergey's Manual of Systematic Bacteriology* (Krieg and Holt, 1984). Any recommendation for re-classification would be complicated at present, however, due to the absence of key phenotypic determinants for each group (Willems *et al.*, 1992), although appropriate genotypic tests could be used. *Pseudomonas fluorescens* is the type strain of the genus (Palleroni, 1984). As the other important species (*Ps. fragi* and *Ps. lundensis*) in meat spoilage are in the same rRNA grouping as the type species, there is little likelihood of these species being removed from the genus.

The pseudomonad consortium occurring on aerobically stored meat is generally dominated by *Ps. fragi* (Table 6.2). *Pseudomonas fluorescens* and *Ps. lundensis* (Molin *et al.*, 1986) were present also in most studies. As yet the reasons for the apparent uncompetitiveness of the last two is unknown. In all but one of the many studies of MAP meats, the proportions of the three species were not determined. The exception was the study by Erichsen and Molin (1981) of isolates from pork of normal pH and stored in a modified atmosphere (78% N_2 + 20% CO_2 + 2% O_2) at 4 °C. *Pseudomonas fragi* comprised only 12% of the microflora; fluorescent strains were not detected. In the present study particular attention was given to the incidence of these three species on MAP meats stored at 0 or 5 °C.

Different numbers of tests have been used to characterise *Pseudomonas* spp. in previous studies. Shaw and Latty (1984) used 18 tests in a study of 787 strains of *Pseudomonas*. Molin and Ternström (1986) used 215 tests to characterise a smaller number of strains (200) in what was effectively a detailed taxonomic study of meat pseudomonads. As a large number of strains needed to be identified in the present study, a small number of tests (Table 6.3) were used to characterise routinely >1250 isolates which had been assigned to the genus *Pseudomonas* on the basis of Gram reaction, cell morphology, oxidative metabolism of glucose and oxidase reaction.

Table 6.2 Prevalance of *Pseudomonas* spp. on meats stored aerobically

	Temperature of storage (°C)	*Pseudomonas* (%) *fragi*	*fluorescens*	*lundensis*
Blickstad *et al.*,1981	4	91	-[c]	-
	14	64	32	-
Erichsen and Molin,1981	4	60	16	-
Molin and Ternström,1982	N.S.[b]	56	13	10.5
Shaw and Latty,1982	1	76	-	16
Banks and Board,1983	4	65	15	-
Blickstad and Molin,1983	0	93	-	-
	4	63	10	4
Shaw and Latty,1984	0, 5, 10	78	4.8	11.1
Molin and Ternström,1986	4	66	14	20
Gennari and Dragotto,1992[a]	5	-	41	44
Prieto *et al.*,1992	7	45	18	15
	30	65	4	21

a By design only fluorescent strains were isolated
b N.S. Not specified
c - Not isolated from the microbial association

Even with the small number of tests, biovars of *Ps. fragi* - but not of *Ps. fluorescens* - could be identified. The *Ps. fragi* groups can be differentiated on the basis of the inositol utilisation test; groups B1->B3 are able to grow with inositol as a sole carbon source whilst *Ps. fragi sensu stricto* cannot (Molin and Ternström, 1986). The tests used in this study were taken from those of the studies of Molin and Ternström (1986) and Molin *et al.* (1986) and their validity established with type cultures. When acceptable differentiation was not achieved, an isolate was characterised using a further eight tests (Table 6.3). In certain trials using MAP meat some strains gave unacceptable profiles. In one case these strains were examined further using 12 tests (Table 6.3). Routine tests were done in a microtitre plate to minimise the labour requirement.

Table 6.3 **Expected profiles for attributes of meat pseudomonads** [a]

	Pseudomonas		
	fluorescens 1/2/3/4 [b] n = 27/5/6/16[c]	***fragi*** B1->B3 */sensu stricto*[b] n = 33/183	***lundensis*** n = 40
Routine tests:			
Acid from maltose	-[d] 4,0,0,0 [e]	+ 100,100	+ 100
Carnitine utilisation	+ 93,100,100,100	+ 92,80	- 0
Inositol utilisation	+ 100,100,100,88	+/- 100,5	+ 90
Additional tests:			
Utilisation of:			
Creatine	- 0,0,17,6	+ 94,97	+ 78
Deoxycholate	- 0,0,0,0	+ 97,95	+ 85
Galactonate	+ 93,100,100,100	+/- 100,13	- 0
Malonate	+ 100,100,100,81	- 0,0	- 0
Quinate	+ 100,100,100,100	+ 100,99	- 2
Saccharate	D 100,100,0,100	+ 100,97	- 2
Xylose	D 85,100,0,44	+ 100,100	- 0
Glucose	+ 100,100,100,100	+ 100,100	+ 100
Gelatin liquefaction	+ 96,80,100,94	v/- 39,10	+ 92

a Source Molin and Ternström (1986) and Molin *et al.* (1986)
b Biovars
c Numbers of each biovar/group in these studies
d - 0-25%, v 26-74% and + 75-100% positive reactions
D Different reactions with each biovar
e Percentage of positive strains of each biovar

MATERIALS AND METHODS

Isolation

At the outset of this study *Pseudomonas* spp. were enumerated on Cephaloridine-Fucidin-Cetrimide agar (CFC, Lab M or Oxoid) after incubation at 25 °C for 2 days. The medium was made according to the manufacturer's instructions. In later studies (insert at pp. 54) a modified CFC - addition of 1% (w/v) L-arginine hydrochloride (Sigma) and 0.002% (w/v) phenol red (BDH) before autoclaving - was used. With both media the antibiotic supplement was added immediately before pouring into Petri dishes. After enumeration five colonies were chosen randomly by drawing a line across the Petri dish and choosing the five colonies closest to the line. Cells were transferred to nutrient agar (NA, Lab M incubated at 25 °C for a maximum of two days) with a sterile wooden applicator, and streaked on two occasions to obtain pure cultures.

Maintenance

Single colonies were transferred from Petri dishes to a NA slope, incubated overnight at 25 °C and kept at 4 °C for medium term storage. For long periods of storage the method of Rhodes (1957) was used; the entire surface of a slope was immersed in sterile liquid paraffin and stored at 4 °C. Each stock was sub-cultured twice on NA before characterisation.

Stock cultures of the type strains of *Pseudomonas fluorescens* biovar 1 (LMG 1794), and *Ps. fragi* (LMG 2191), a strain of *Pseudomonas fluorescens* biovar 3 (LMG 5822), and *Ps. lundensis* isolated from beef steak and identified at Bath University were maintained as described above and used alongside recent isolates to validate the methodologies.

Characterisation

The isolates were tested for Gram reaction, cell morphology, oxidase reaction and the production of acid from glucose (modified Hugh and Leifson medium, Harrigan and McCance, 1976). Gram negative, oxidase positive rods which produced acid from glucose by oxidative metabolism were tentatively assigned to *Pseudomonas.*

Three tests were used for further characterisation of *Pseudomonas*, namely the production of acid from maltose, and the utilisation of carnitine and inositol as sole carbon sources (tetrazolium violet as indicator). During the development of these methods, a basal

medium containing tetrazolium violet but no carbon source was included. On no occasion was there a colour change (colourless to violet) in the absence of a substrate. Glucose as a carbon source was always included as a positive control. The media used for these tests were (all % w/v unless otherwise stated):

ACID FROM MALTOSE:

Peptone	0.2
NaCl	0.5
KH_2PO_4	0.03
Bromocresol Purple	0.006
Maltose	1.0

CARBON UTILISATION

Na_2HPO_4	0.3
KH_2PO_4	0.23
NH_4Cl	0.1
$MgSO_4.7H_2O$	0.05
Ferric Ammonium Citrate	0.005
$CaCl_2$	0.0005
Tetrazolium Violet (T.V.)	0.01
Trace element stock solution (see below)	1v/v
Carbon source (filter sterilised)	0.2

Trace element stock solution % w/v: H_3BO_3 0.289, $MnCl_2.4H_2O$ 0.181, $ZnSO_4.7H_2O$ 0.022, $Na_2MoO_4.2H_2O$ 0.039, $CuSO_4.5H_2O$ 0.008, $Co(NO_3)_2.6H_2O$ 0.005

The media were made up at a 1.3 times concentration - the substrates required for 100ml of medium were dissolved in 75ml of water - and sterilised by autoclaving (121 °C for 15 minutes). In carbon utilisation tests precipitation during autoclaving led to the following regime being adopted.

The trace element solution was added to the required volume of water (allowing for the volume of the stock solutions to be added later) and sterilised by autoclaving. The two phosphates were combined in 50X stock solution and filter sterilised. The tetrazolium violet was made as a 100X stock solution and autoclaved. The carbon sources were made up as 100X stock solutions and filter sterilised; all the other components were combined and treated in the same manner as the carbon source. All stock solutions were added before use.

The final media were dispensed into the wells of a microtitre plate (150µl per well). An inoculum was prepared by transferring a small amount of growth from an overnight culture of the isolate on nutrient agar to 1ml of sterile water in an Eppendorf tube. Fifty microlitres of the cell suspension were transferred to each well of the microtitre plate. This diluted the medium to the working concentration. The plates were incubated at 25 °C for

up to 7 days. Acid production from maltose (bromocresol purple gives purple to yellow colour change) and carbon utilisation (tetrazolium violet - colourless to purple) were scored as positive (Figure 6.1).

The gelatin liquefaction test used in the extended analysis was done in bijou bottles. Three ml of nutrient gelatin (100 ml nutrient broth + 15g gelatin) were distributed into bijou bottles, autoclaved (121 °C for 15 minutes) and cooled. The medium was stab inoculated and incubated at 25 °C. Gelatin liquefaction was checked after 7 and 14 days, the bottles being cooled in a refrigerator before recording the results.

Reproducibility

Ten % of the strains in each trial were re-tested to determine the reproducibility of the tests. The following equation was used to give a reproducibility percentage:

$$\frac{\text{Number of tests giving the same reaction}}{\text{Total number of tests}} \times 100$$

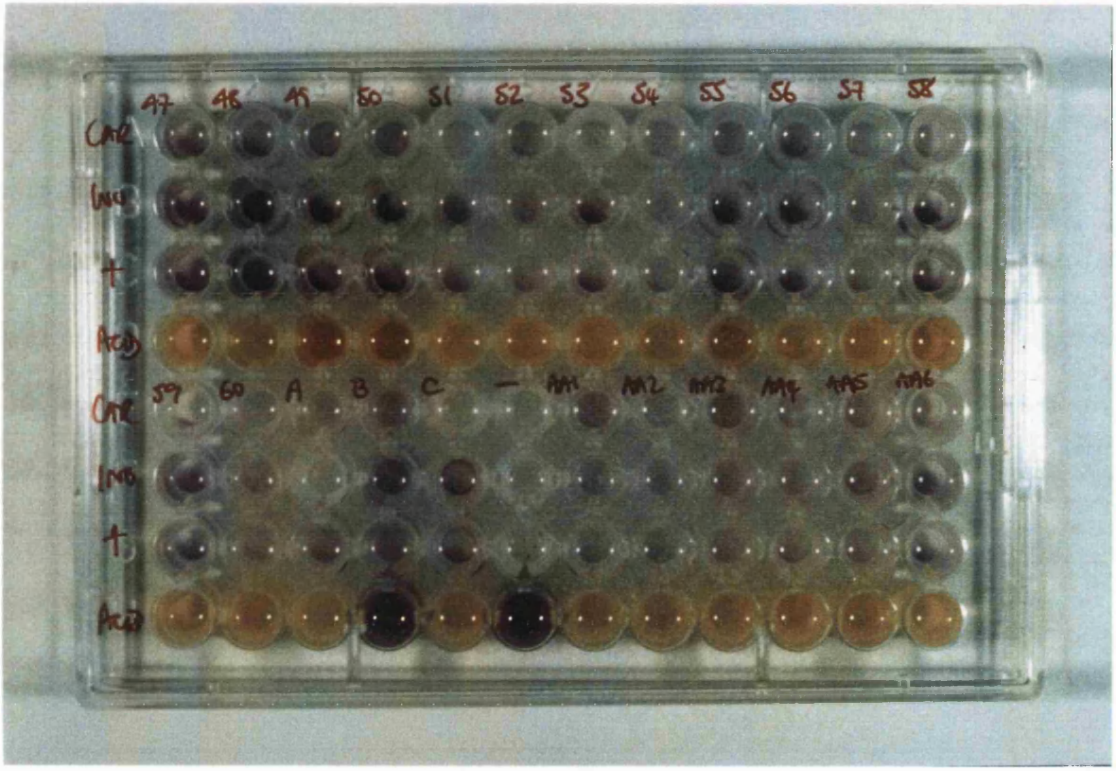


Figure 6.1 **Characterisation of *Pseudomonas* spp. using a microtitre plate method**

A	*Ps. fragi*
B	*Ps. fluorescens*
C	*Ps. lundensis*
Other strains	Test strains isolated from a variety of meat samples
CAR	Carnitine utilisation (colour change - colourless to purple with growth)
INO	Inositol utilisation (colour change - colourless to purple with growth)
+	Glucose utilisation (colour change - colourless to purple with growth)
ACID	Production of acid from maltose (colour change - purple to yellow with acid)

RESULTS

The incidence of the three species of *Pseudomonas - fragi, fluorescens and lundensis* - developing on beef steaks stored in a variety of atmospheres at 0 or 5 °C was studied, the organisms being isolated from the selective medium, CFC. Over 1950 isolates were purified; of these, 1252 were identified to species level. Of the remainder, only a few were unidentified and the majority were found to be "contaminating" bacteria - mainly members of the family Enterobacteriaceae with some *Acinetobacter/Moraxella* -and yeast. Many of the unidentified organisms were isolated on standard CFC as used in the first trial (meat deliberately inoculated with *Listeria monocytogenes*). It was surmised that the selectivity of the medium was taxed during the long period that the Petri dishes were stored before delivery to Bath and subsequent isolation. As this situation obtained throughout the trial with meat inoculated with *Salmonella typhimurium* (Salmonella I), it was evident that there was a fundamental problem requiring amendment of CFC. A modified medium was developed (insert to pp. 54) and used in the Salmonella II trial. The numbers of pseudomonads isolated relative to contaminating micro-organisms were increased significantly because the Enterobacteriaceae could be differentiated easily from the pseudomonads.

The overall proportions of the three species of *Pseudomonas* isolated in all the trials are shown in Table 6.4. It is evident that *Pseudomonas fragi* dominated the pseudomonad population on beef steaks in MAP. Its proportion varied in different trials, however, being much lower (45% *vis à vis* >64%) in the Salmonella II than in the other trials. The incidence of the other two species were generally of the same magnitude (Tables 4.2 and 4.4). The species-incidence in data from all the trials was similar to that in the many studies of aerobically stored meats (Table 6.2).

Having demonstrated the overall dominance of *Pseudomonas fragi* in MAP, the effect of different storage conditions on the pseudomonad populations was examined. The packed beef steaks were stored at 0 or 5 °C under vacuum packs (VP), in 50% N_2 + 50% CO_2 (50/50), 80% O_2 + 20% CO_2 (80/20) or 100% CO_2 (CO_2). As determined by enumeration on CFC, the growth of pseudomonads was generally inhibited by all the adopted atmospheres. The microflora on MAP meat was dominated by lactic acid bacteria either alone or in combination with *Brochothrix thermosphacta* (see Chapter 2). In other words the increase in numbers of pseudomonads in any of the atmospheres at either temperature was slight in comparison with the lactic acid bacteria. Thus, there was a 2 log cycle increase of CFC

Table 6.4 Prevalence of *Pseudomonas* spp.* on beef steaks stored in modified atmospheres[a]

Trial	Temperature of storage (°C)	*Pseudomonas* (%)		
		fragi	*fluorescens*	*lundensis*
Listeria[b]	0	64.4	19.6	16.0
	5	69.5	16.8	13.2
Salmonella I	0	68.0	9.9	22.0
	5	75.0	9.9	15.1
Salmonella II	5	45.0	24.0	31.0
Total		63.7	16.9	19.2

* *Pseudomonas putida* was isolated on two occasions only

a Vacuum packs, 50% N_2 + 50% CO_2, 80% O_2 + 20% CO_2, 100% CO_2

b Beef steaks were deliberately inoculated with the named pathogens, stored in MAP at chill temperatures and the microbial population analysed. Isolates from the *Pseudomonas* selective medium were identified.

counts in 80/20 at 0 °C. At 5 °C the inhibition of these organisms was less pronounced, there being a 3 log increase in 80/20 and a 2 log differences in 50/50 and in VP. The numbers of pseudomonads remained unchanged throughout storage of meat in CO_2.

Even though there were only small changes in the overall population size of pseudomonads with storage, there could have been marked differences in the behaviour of the three species. It is important to realise that even a very small portion of a microbial association may produce spoilage odours, a point emphasised by Dainty and Mackey (1992). Thus the species changes throughout storage must be evaluated. Three trials were done during the course of this study. The reliability of the sampling methodology was determined in the Listeria trial. Strains were isolated from duplicate packs for each atmosphere and temperature at every sampling time. The steak in a pack was quartered and two quarters sampled. The numbers of bacteria growing on CFC were not significantly different between either packs or the two quarters sampled from an individual pack (A.R. Davies, pers. comm.). The numbers of micro-organisms identified in the different samples are shown in Table 6.5. As there were no significant differences between the quarters within a pack, duplicate packs only were sampled in subsequent trials. For every atmosphere at each temperature, twenty isolates were taken in the initial trial (Listeria); with the two Salmonella trials, ten isolates were identified for each storage condition at each time of sampling.

The results of various trials are presented in Figures 6.2 (0 °C) and 6.3 (5 °C). Only the initial proportions and those at the end of the storage period (usually coincident with the onset of spoilage) for each atmosphere are shown. The changes occurring with time are given for the Salmonella II trial at 5 °C (Figure 6.4). There are no data for 0 °C for the second Salmonella trial; samples stored at 5 or 12 °C were enumerated but only bacteria from the

former were identified. In the first two trials (Listeria and Salmonella I), *Ps. fragi* dominated the population almost to the total exclusion of the other two species in the majority of the atmospheres at 0 and 5 °C. There was an exception at each temperature. The atmosphere containing CO_2 only in the Salmonella I trial at 0 °C and the vacuum pack in the Listeria trial at 5 °C being the exceptions. In both cases the proportions of *Ps. lundensis* equalled those of *Ps. fragi.* The second Salmonella trial gave a different pattern. *Pseudomonas fragi* was apparently inhibited completely by atmospheres containing high concentrations of CO_2 (50/50 and CO_2). In the vacuum pack the numbers of *Ps. lundensis* were again equal to those of *Ps. fragi* and it was only in the high oxygen atmosphere (80/20) that the latter was dominant. Such differences between trials are expected if the proportion of each species present in the initial population is considered. In the first two trials *Ps. fragi* was the dominant pseudomonad at the outset, whereas in the Salmonella II trial *Ps. fluorescens* contributed > 50% of the initial pseudomonad flora, with the proportion of *Ps. fragi* being reduced to <10% at the end of storage.

These differences were reflected also in the proportions of *Ps. fluorescens* which persisted throughout the storage period. At 0 and 5 °C *Ps. fluorescens* formed a minor portion of the flora except in the second Salmonella trial (5 °C) in the high CO_2 atmospheres. It was dominant in the atmosphere containing CO_2 only and was present in numbers equal to those of *Ps. lundensis* in the 50/50 atmosphere. Although it formed co-dominant populations (noted above), *Ps. lundensis* was never the numerically dominant pseudomonad at the end of storage,

During storage there were a number of changes in the proportion of the three species (Figure 6.4). The numbers of *Ps. lundensis* tended to increase towards the end of storage (Figure 6.4a and 6.4c) except with the 80/20 and CO_2 atmospheres where a peak in its numbers obtained before the end of storage. Some of these results may be distorted by the low numbers of pseudomonads available for isolation. An increase followed by a decrease in the percentage of the population was seen with *Ps. fragi* in vacuum packs and in the 50/50 atmosphere. *Pseudomonas fluorescens* gave a peculiar trend with the 50/50 atmosphere. It was present as a co-dominant in the population (50%) on the last day of sampling (day 35) but was not isolated from the two preceding samples. Again there were small numbers of organisms present on days 28 and 35.

These trials demonstrated conclusively that pseudomonads were unable to grow extensively in MAP beef steaks. The fact that growth to a limited extent occurred reveals that none of the selected atmospheres was completely inhibitory.

Table 6.5 The variation in the pseudomonad population within and between packs at the end of storage for the Listeria trial

	Vacuum Pack				50% O_2 + 50% CO_2				80% O_2 + 20% CO_2				100% CO_2			
Samples:[a]	1		2		1		2		1		2		1		2	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Day of sample	70	70	70	70	56	56	70	56	56	56	56	56	70	43	56	43
Ps. fragi B [b]	**2****	**1**	**1**	**2**	**2**	**1**	**5**		**3**			**3**	**4**	**4**	**2**	**3**
fragi s.s.		**2**	**3**	**3**	**1**				**1**					**1**		**1**
fluorescens					**1**										**1**	
lundensis																
Oxidase -ve	**3**	**2**	**1**			**1**										
Yeasts						**3**		**5**		**5**	**5**	**1**	**1**		**1**	

a) Uninoculated packs stored at 0 °C

	Vacuum Pack				50% O_2 + 50% CO_2				80% O_2 + 20% CO_2				100% CO_2			
Samples: [a]	1		2		1		2		1		2		1		2	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Day of sample	77	77	77	49	77	63	63	63	49	49	49	49	49	77	49	49
Ps. fragi B			**1**		**1**				**1**	**1**	**5**	**2**	**4**	**1**	**2**	**1**
fragi s.s.		**1**													**1**	
fluorescens								**2**	**4**	**4**		**3**				
lundensis	**2**	**1**		**1**												
Oxidase -ve	**3**	**3**	**4**	**2**	**2**	**5**	**1**						**1**			
Yeasts														**3**		

a 1 and 2 correspond to duplicate packs, A and B correspond to duplicate quarters within a pack

b *Ps. fragi* B - group B1 -> B3 as described by Molin and Ternstrom (1986)
- s.s. *sensu stricto*
Oxidase -ve - Gram negative rods which do not give a positive oxidase reaction

** Numbers of strains identified

b) Inoculated packs stored at 0 °C

Table 6.5 contd. **The variation of the pseudomonad population within and between packs at the end of storage for the Listeria trial**

	Vacuum Pack				50% O_2 + 50% CO_2				80% O_2 + 20% CO_2				100% CO_2			
Samples:[a]	1		2		1		2		1		2		1		2	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Day of sample	34	34	34	34	29	29	29	29	29	29	29	28	28	28	28	28
Ps fragi B [b]		1**	1						5	4	1	3	2		1	
fragi s.s.													1			
fluorescens										1	3	1				
lundensis			2								1	1				
Oxidase -ve	5	3	1	3	5	5	5	5					2	5	3	4*
Yeasts																

c) Uninoculated packs stored at 5 °C

	Vacuum Pack				50% O_2 + 50% CO_2				80% O_2 + 20% CO_2				100% CO_2			
Samples:[a]	1		2		1		2		1		2		1		2	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Day of sample	34	34	34	34	24	24	24	24	24	24	24	24	21	21	21	21
Ps fragi B				2					5	3	3	3				
fragi s.s.	1		2		1									1		1
fluorescens								1		1	1	1				
lundensis	3	1	1	1								1	1	1		
Oxidase -ve	1	3	2	2	2	5	5	4		1	1		4	3	5	3
Yeasts																

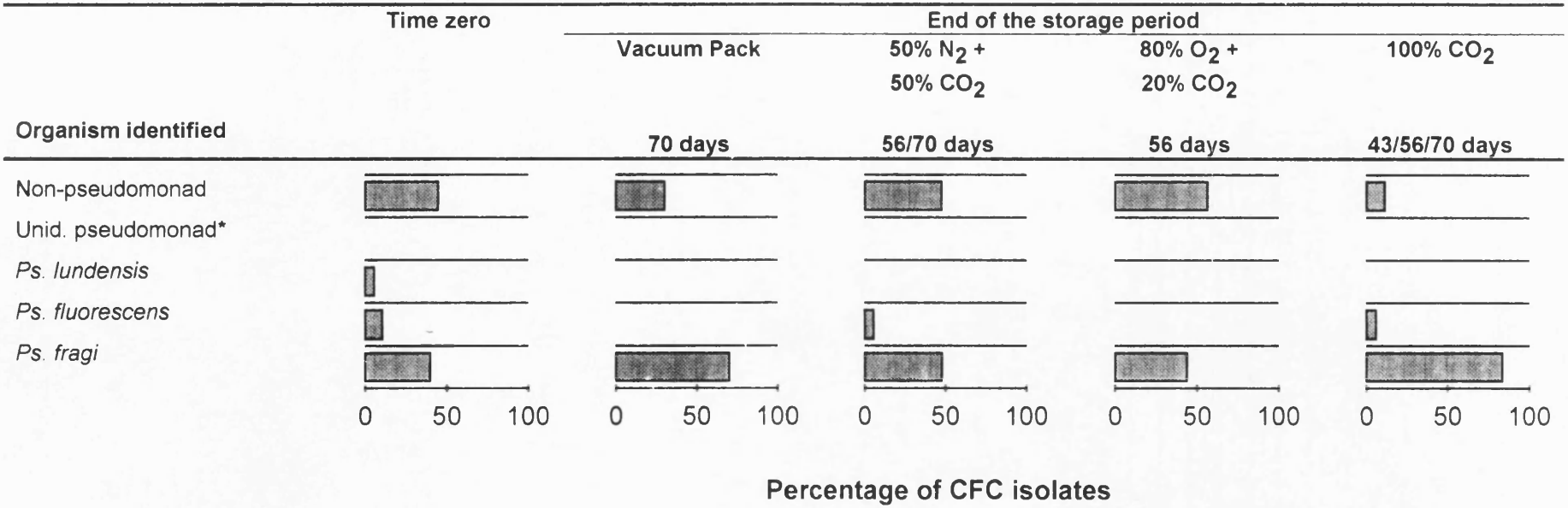
a 1 and 2 correspond to duplicate packs, A and B correspond to duplicate quarters within a pack

b *Ps. fragi* B - group B1 -> B3 as described by Molin and Ternstrom (1986)
- s.s. *sensu stricto*
Oxidase -ve - Gram negative rods which do not give a positive oxidase reaction

* *Acinetobacter*

** Numbers of strains identified

d) Inoculated packs stored at 5 °C

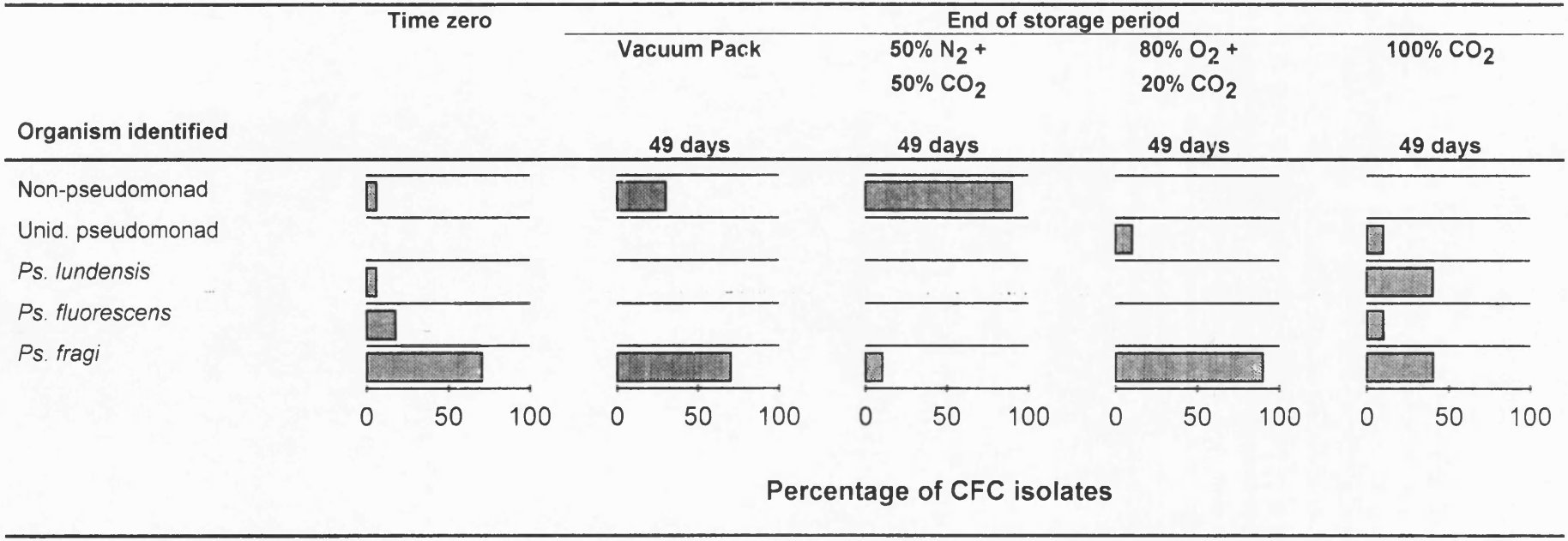


Reproducibility of test method - 86.4%. Tetrazolium violet was not used during this trial. The indicator was added to improve the recording of the result and hence reproducibility.

* Unid. Unidentified

a) Listeria trial

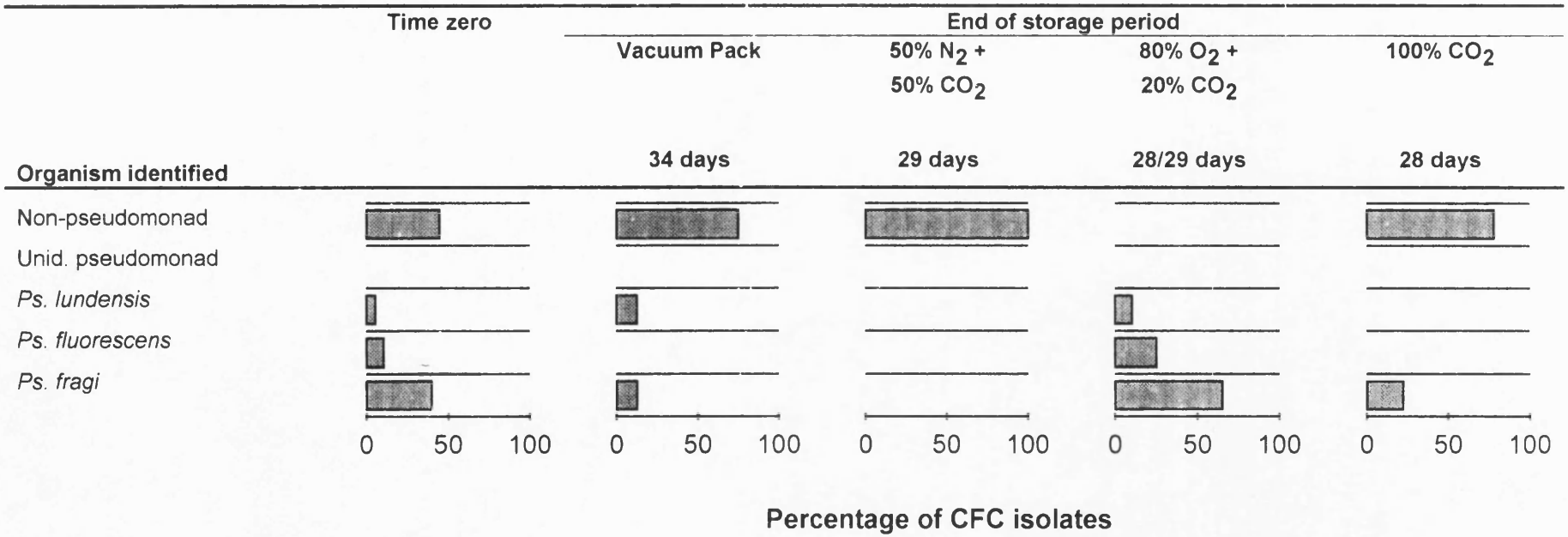
Figure 6.2 **Identification of *Pseudomonas* spp. in modified atmosphere packaged beef steaks stored at 0 °C**



Reproducibility of test method - 96.1%

b) Salmonella I

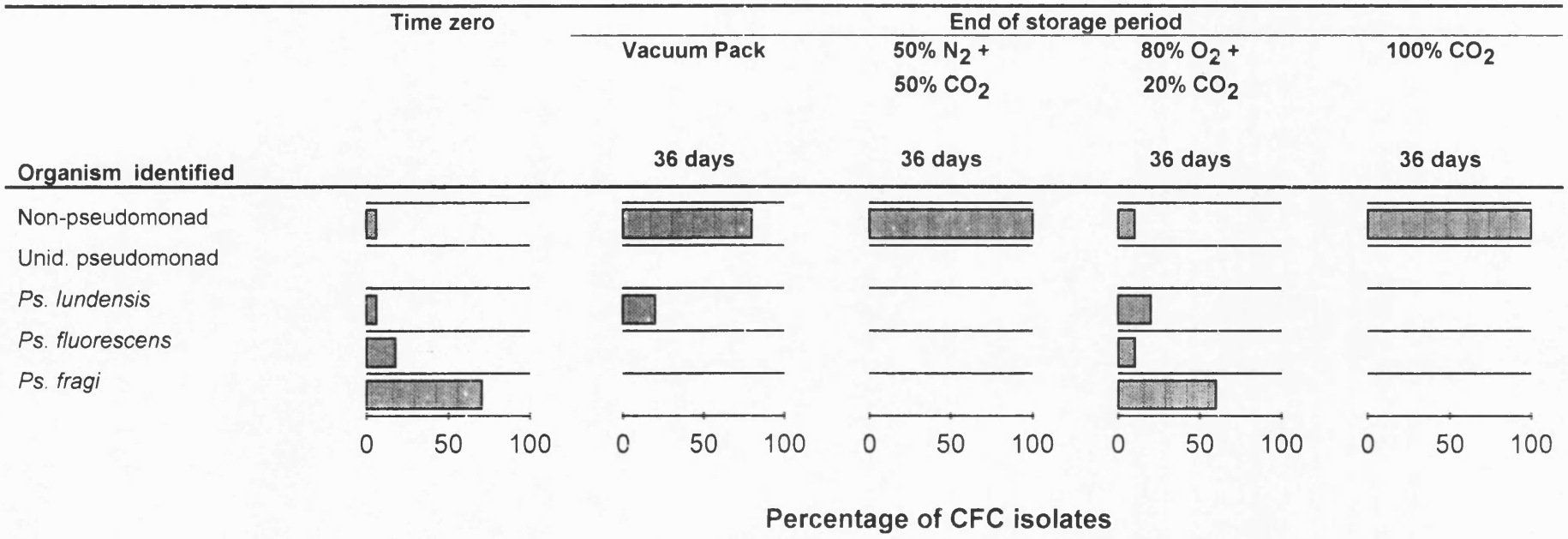
Figure 6.2 contd. **Identification of *Pseudomonas* spp. in modified atmosphere packaged beef steaks stored at 0 °C**



Reproducibility of test method - 86.4%. Tetrazolium violet was not used during this trial. The indicator was added to improve the recording of the result and hence reproducibility.
* Unid. Unidentified

a) Listeria trial

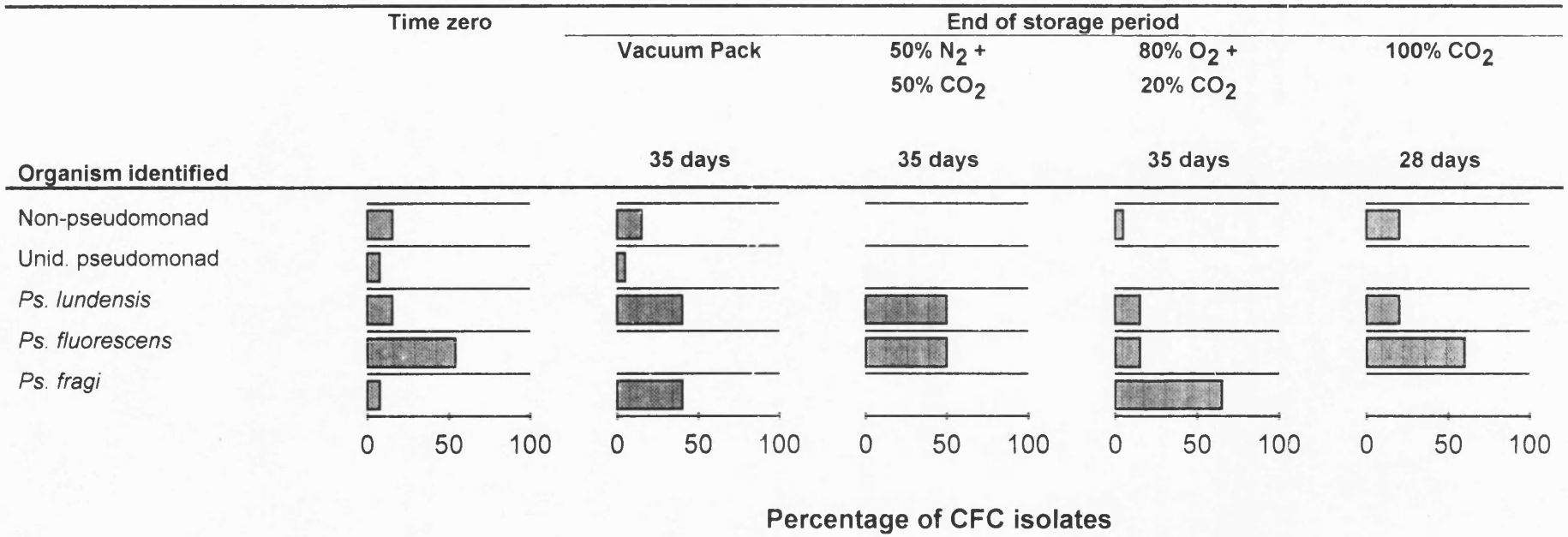
Figure 6.3 **Identification of *Pseudomonas* spp. in modified atmosphere packaged beef steaks stored at 5 °C**



Reproducibility of test method - 96.1%

b) Salmonella I trial

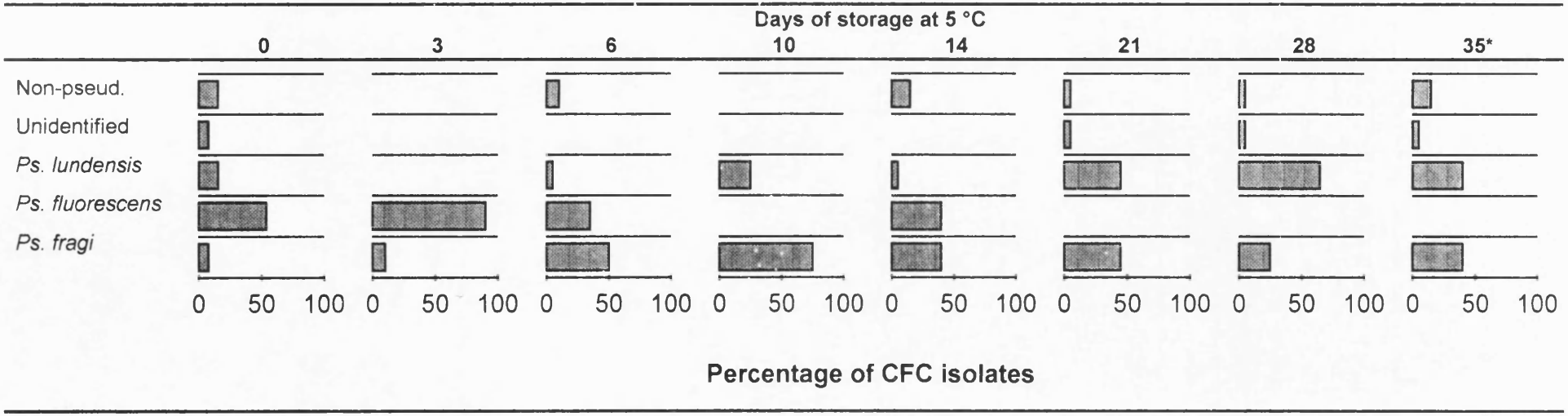
Figure 6.3 contd. **Identification of *Pseudomonas* spp. in modified atmosphere packaged beef steaks stored at 5 °C**



Reproducibility of test method - 94.7%

c) Salmonella II trial

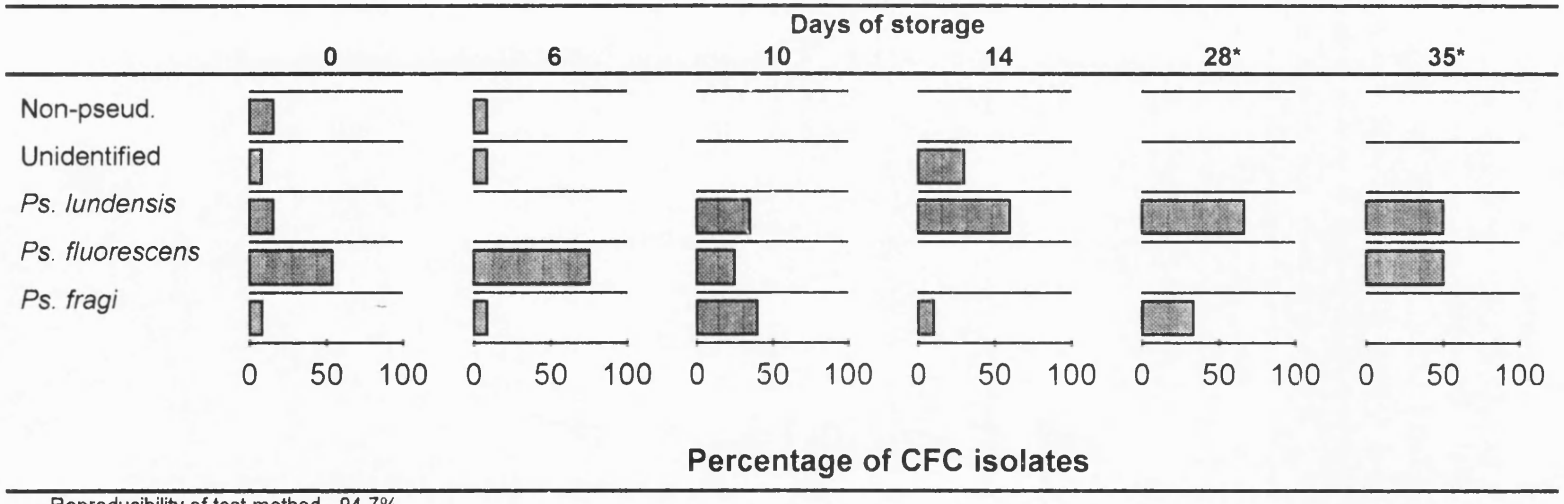
Figure 6.3 contd. Identification of *Pseudomonas* spp. in modified atmosphere packaged beef steaks stored at 5 °C



Acinetobacter not G -ves
Reproducibility of test method - 94.7%

a) Vacuum pack

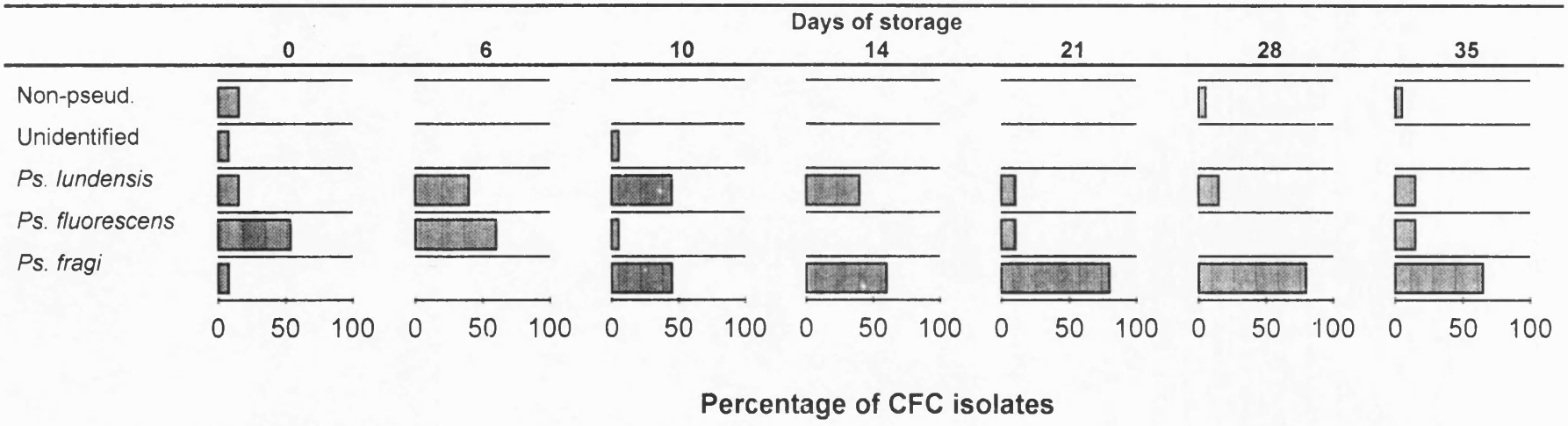
Figure 6.4 **Changes in the pseudomonad population on modified atmosphere packaged beef steaks stored at 5 °C - Salmonella II trial**



Reproducibility of test method - 94.7%

b) 50% N_2 + 50% CO_2

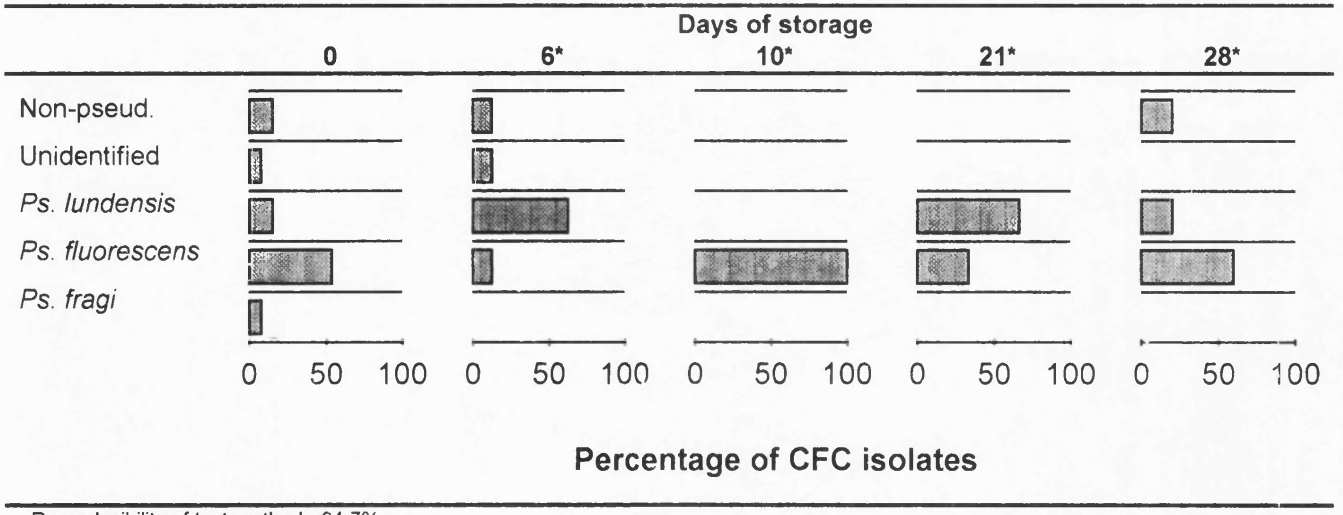
Figure 6.4 contd. **Changes in the pseudomonad population on modified atmosphere packaged beef steaks stored at 5 °C - Salmonella II trial**



Reproducibility of test method - 94.7%

c) 80% O_2 + 20% CO_2

Figure 6.4 contd. **Changes in the pseudomonad population on modified atmosphere packaged beef steaks stored at 5 °C - Salmonella II trial**



Reproducibility of test method - 94.7%
* Very low numbers in samples

d) 100% CO_2

Figure 6.4 contd. **Changes in the pseudomonad population on modified atmosphere packaged beef steaks stored at 5 °C - Salmonella II trial**

DISCUSSION

No large scale analysis of the pseudomonad populations on MAP meats has been done previously. The methodology devised for the identification of pseudomonads from modified atmosphere packaged meats proved effective. The selected tests gave a rapid and reproducible characterisation and hence identification of very large numbers of isolates (>1250). There were occasions when the profiles were not consistent with predicted results, possibly due to the presence of strains with atypical characteristics. A small number of isolates were identified with *Pseudomonas* but their profiles prevented species identification. Cox and MacRae (1989) isolated pseudomonads from goats milk; some isolates had phenotypic attributes intermediate of those of known species. Such strains may be as yet undefined species. This is unlikely in view of the results from strains from the Salmonella II trial. These suggested that some of the "atypical" profiles given by the three tests may well be those of additional biovars of *Ps. fragi.* Further work is needed to resolve the taxonomy of these and other unidentified strains. As strains in MAP meat would be present on meat in abattoirs, their isolation would have been expected in previous studies of this environment (e.g. Gill and Bryant, 1992; Gustavsson and Borch, 1993). Perhaps selection of relatively few organisms for study by these authors excluded unusual types. Isolation of different species would be more likely of course if selective media other than CFC, alternative incubation conditions, or both, had been used. Such an approach may support the growth of previously unknown organisms.

Until the introduction of CFC, there was no satisfactory medium for the isolation of pseudomonads that were not dominant in an environment. Although CFC has been widely used it is evident that it supports the growth of organisms other than pseudomonads. Indeed Edwards and Dainty (1987) isolated micro-organisms on CFC from vacuum packaged pork of normal and high pH and identified them with *Shewanella (Alteromonas) putrefaciens* (orange/brown colonies) or with members of the Enterobacteriaceae. In practice, the bacterial counts on CFC medium were of the same order as those on Violet Red Bile Glucose agar, the Enterobacteriaceae selective medium.

It must be stressed that in MAP meats stored at chill temperatures the *Pseudomonas* spp. multiply to a limited extent (maximum increase of 2 log cycles at 0 °C and 3 log cycles at 5 °C). They did not, however, form populations which contributed significantly to the microbial association on the beef steaks. These organisms flourish without resuscitation once exposed to an aerobic atmosphere even on selective media. This underlines two

features of commercial importance of MAP meats. One is the importance of preventing leaks in packs intended for long term storage. The other relates to the treatment of meat removed from MAP - pseudomonad spoilage would result from extended storage after unpacking.

As stressed previously, the temperature of storage is of paramount importance. The inhibitory effect of CO_2 diminishes with increasing temperature. Thus in this study the growth of pseudomonads at 5 tended to be greater than that at 0 °C. The temperature abuse of packs may also lead to the growth of these spoilage organisms.

The pattern of population dynamics described above is similar to that of pseudomonads in soil. Members of this genus commonly (3 - 15%) are a part of the zymogenous portion of the soil population (Holding, 1960). There are intermittent periods of high activity (rapid growth) associated with nutrient input followed by long periods of inactivity where numbers decline rapidly to the resident level. In the quiescent periods most of the energy of a cell is needed for maintenance of the essential cellular processes such as osmotic regulation and maintenance of intracellular pH. It would be expected that breakdown of RNA and proteins would be a function of the quiescent state (Dawes, 1976). Similar events may be expected to occur in the meat environment, but as yet the nature of the energy status of pseudomonad cells in MAP meat storage has not been determined.

Pseudomonas fragi has been shown to be the dominant pseudomonad on aerobically stored meats (see Table 6.2) with *Ps. fluorescens* and *Ps. lundensis* generally occurring as minor parts of the microflora. Few studies have been done, however, to analyse changes within a pseudomonad population during storage. Erichsen and Molin (1981) showed that the population of pseudomonads changed from the beginning to the end of storage of pork. Only the non-fluorescent strains were present after storage in a gas mixture of 78% N_2 + 20% CO_2 + 2% O_2. These comprised 12% of the total microflora on normal pH (87% initially) and 44% on high pH meat (18% initially). Fluorescent pseudomonad strains identified with *Ps. fluorescens* were not detected in their study. As would be expected, lactic acid bacteria dominated the population in the MAP packs of meat of normal pH, and in vacuum packs and 100% CO_2 packs containing both types of meat. These results were similar to those obtained in the present study. *Pseudomonas fragi*, the non-fluorescent species, was generally the dominant pseudomonad in the population developing on meat of normal pH.

The results of the present study revealed differences in the level of inhibition of the three meat pseudomonads by temperature and atmosphere. The effect of temperature on

the growth rate of pseudomonads has been known for many years. Haines (1933a) determined generation times of 7.9 and 1.1 hours for a *Pseudomonas* sp. in air at 4 and 20 °C respectively. After a protracted lag period in an atmosphere enriched with 20% CO_2 the times were 21.8 and 1.6 hours at 4 and 20 °C respectively. Gill and Tan (1980) found that CO_2 inhibition of a non-fluorescent pseudomonad was less than that of a fluorescent strain under the same conditions. The stimulation of the growth of *Ps. fluorescens* by low levels of CO_2 (Gill and Tan, 1979) suggests that complex reactions occur during the growth of the organism in this "inhibitory" gas. As single strains of each group only were studied, it was emphasised that generalisations should only be made with the utmost care. The actual cause of inhibition is unknown although Tan and Gill (1982) implicated a non-specific inhibition of substrate uptake. It has been shown that there is preferential use of certain substrates under different storage conditions (Molin, 1985), with lactate rather than glucose being the first substrate to be utilised under oxygen limited conditions. In contrast, glucose uptake is enhanced in the presence of CO_2. The effects of these gases on particular enzymes (e.g. protease, lipase, enzymes for glucose metabolism) have been studied in *Ps. fluorescens* and *Ps. aeruginosa* (Rowe, 1988; Mitchell and Dawes, 1982). Again the studies were done on single strains in artificial media so comparisons of the results to the situation in a meat environment can not be made.

Indeed, the limitation of oxygen may be as important as the presence of carbon dioxide. The study by Molin (1983) revealed that with *Ps. fragi* in continuous culture, oxygen limitation had a greater effect than inhibitory levels of CO_2 on population size. A combination of the two indicated also that synergism may be involved. A large drop in growth yield of *Pseudomonas aeruginosa* with oxygen limitation was also noted by Mitchell and Dawes (1982). The differences in the composition of pseudomonad flora in various storage conditions tested may be due to a preferential uptake and use of available substrates, with the carbon dioxide inhibition and oxygen limitation affecting the enzyme action of both the uptake and metabolic pathways.

There may be other factors involved in the selection of the meat pseudomonads in different storage conditions. Fourteen of 209 strains of *Pseudomonas* isolated from fish were found to inhibit *Ps. fluorescens* (Gram, 1993). It is possible, therefore, that a similar situation pertains in meat, with individual strains producing inhibitory agents. A study was done using 211 pseudomonads which were isolated from meat as potential inhibitors to other meat strains (Champomier and Richard, 1994). None of the former were found to produce inhibitors to test strains. A number of fluorescent pseudomonads isolated from fish

were able to inhibit *Ps. fragi* (isolated from meat), possibly due to the action of a siderophore (Champomier and Richard, 1994). Other bacteria present in the microflora may also play a part in the selection of certain pseudomonads. Some lactic acid bacteria are known to inhibit pseudomonads through the production of hydrogen peroxide (Price and Lee, 1970). The former group have been shown also to produce other antimicrobial agents, particularly organic acids and bacteriocins (Piard and Desmazeaud, 1991a,b). *Pseudomonas fragi* growth was inhibited by 0.5% lactic acid (Ziauddin *et al.*, 1993), although there was no differentiation of the effect of the organic acid *vs* pH of the broth - pH 5.0.

CHAPTER 7
GENERAL DISCUSSION AND FUTURE WORK

Almost 3,000 bacteria were purified from selective and elective media and subsequently identified during the course of the present study. As such, the populations developing on MAP beef stored at 0 or 5 °C were analysed in detail. The numerically important members of the microflora were identified.

The modification of the atmosphere generally inhibited the growth of pathogenic micro-organisms (Table 3.5) *vis-à-vis* on aerobically stored meats. This was particularly so in the case of beef, the meat studied during the present work. No significant growth was detected for any of the pathogens inoculated onto beef steaks which were subsequently packaged in MAP and stored at 0 or 5 °C. Packs subjected to the severe abuse temperature of 12 °C did show some growth of pathogens (Table 3.5), but this increase in numbers was never greater on MAP beef than on similar meat stored aerobically (A.R. Davies, pers. comm.).

In this study lactic acid bacteria were found to be numerically dominant on MAP beef steaks stored at 0 or 5 °C. This was in keeping with previous studies (Table A1.1, Appendix 1). Of the LAB on MAP steaks stored at 5 °C *Carnobacterium divergens* was present in the highest proportions. Many previous studies have found that *Lactobacillus sake/curvatus* predominated (Table 4.1, pp. 84), but Hitchener *et al.* (1982) isolated 65% heterofermentative rods and 10% *Leuconostoc mesenteroides* from vacuum packed beef; the remaining 25% were homofermentative rods. The species isolated from the meat ecosystems are affected greatly by the type of meat, packaging method, and of course, the medium used for their isolation. An ability to grow in high CO_2 concentrations favours the growth of LAB *vis-à-vis* Gram negative micro-organisms in the modified atmospheres. It was also noted that this gas affected the microbial species growing/surviving on the meat. Lactobacilli and carnobacteria became the dominant organisms on the steaks in vacuum packs or in 50% N_2 + 50% CO_2, *Leuconostoc gelidum* was dominant in 100% CO_2 and *Brochothrix thermosphacta* grew to the exclusion of all the LAB species in the high oxygen atmosphere (80% O_2 + 20% CO_2). Atmospheres containing high levels of CO_2 inhibited *Ps. fragi* more than the other two meat pseudomonads, *Ps. lundensis* and *Ps. fluorescens*. *Hafnia alvei* was the dominant member of the Enterobacteriaceae, particularly with storage at 5 °C. It was inhibited by high levels of CO_2, but survived in vacuum packs.

The presence of acid and an associated low pH are also important in determining the composition of the microflora developing on MAP meats. This has been discussed previously in relation to the effects on the development of lactic acid bacteria (Chapter 4). The relatively low pH (5.4-5.5) of fresh meat undoubtedly affects the development of the microflora in general. Meat of high pH (pH 6.0) tends to spoil more rapidly (see Table A1.1, Appendix 1) and harbours a different climax population to that of low pH. This difference is reflected in the type of populations developing on beef *vis-à-vis* on lamb

(see Table A1.1, Appendix 1) which tend to have a higher ultimate pH. Beef steaks of high pH packaged under vacuum were found to have 10- to 100-fold higher numbers of Enterobacteriaceae, *Brochothrix* and pseudomonads than on corresponding low pH meat packaged in the same manner (Rousset and Renerre, 1991). Enterobacteria were also found in higher proportions on VP rather than CO_2-packed meat (Gill and Penney, 1986). In beef steaks packaged in 78% N_2 + 20% CO_2 + 2% O_2, the flora was dominated by pseudomonads on high pH and by lactic acid bacteria on low pH meat (Erichsen and Molin, 1981). Vacuum packed dark, firm, dry meat was shown to harbour a flora containing *Yersinia enterocolitica*, *Enterobacter liquefaciens* and *Alteromonas* (now *Shewanella*) *putrefaciens* (Gill and Newton, 1979), organisms found rarely on low pH VP meats. These examples indicate the variation obtained by packaging in different conditions and with high and low pH meats.

The addition of organic acids was shown to greatly affect the development of the microbial groups on VP meat (Witt, 1993; Appendix 3, this thesis). Lactic acid was added to minced rump of beef which was VP and stored at 5 °C. The microbial groups showed different sensitivities to the presence of this acid with *Brochothrix thermosphacta* > pseudomonads and Enterobacteriaceae > lactic acid bacteria (Witt, 1993). A similar, though more marked effect, was noted with the addition of acetic acid (Appendix 3). The numbers of LAB diminished with storage. *Lactobacillus sake* survived after two weeks of storage at 5 °C whereas other LAB did not (Appendix 3).

Lactobacilli are known to tolerate highly acidified environments - pH 3.5 (Kashket, 1987; Nannen and Hutkins, 1991). As discussed previously (Chapter 4), acetic acid has been shown to increase throughout the storage of MAP meats (Kakouri and Nychas, 1994; Drosinos, 1994). The acetic acid produced by heterofermentative LAB, particularly leuconostocs and carnobacteria, may have a fundamental effect on the development of the microflora in environments where these organisms are present in high proportions, *viz.* MAP meats.

Antagonistic agents may also be produced by lactic acid bacteria. These include bacteriocins (discussed in Chapter 4), carbon dioxide, hydrogen peroxide, diacetyl, and acetaldehyde (de Vuyst and Vandamme, 1994). The importance of these inhibitory agents in the development of the microflora in MAP meat environments has generally not been elucidated.

Hydrogen peroxide has an antimicrobial action. It has a strong oxidising effect and may cause destruction of nucleic acid and protein structure (de Vuyst and Vandamme, 1994). Eleven strains of *Pseudomonas* spp. were inhibited *in vitro* by H_2O_2 produced by a *Lactobacillus* strain (Price and Lee, 1970). A strain of *Ps. fragi* was inhibited by H_2O_2 from *Lact. acidophilus* grown at 4 °C, but only when the culture was shaken continuously. During nine days growth, *Ps. fragi* reached numbers of $<10^7$ cfu ml^{-1} *vis-à-vis* $>10^9$ cfu ml^{-1} in the presence of catalase (Collins and Aramaki, 1980).

Some lactic acid bacteria are able to produce diacetyl from citrate or pyruvate and small quantities may also be produced from hexoses (de Vuyst and Vandamme, 1994).

The antimicrobial activity of diacetyl in ethanolic solution was elucidated by Jay (1982). In general, this compound was more effective against Gram negative than Gram positive organisms (Table 7.2). Montville *et al.* (1987) showed that *Lact. plantarum* produced >350 μg ml^{-1} from 100 mM pyruvate in a culture medium. This would be sufficient to have a significant inhibitory effect if the activity of the compound was as effective in meat ecosystems as in ethanolic solution. The production of diacetyl and its potential as an antimicrobial agent in such environments is unknown. The addition of significant concentrations may affect meat flavour as it is an important flavour compound in butter and cheese.

Thus many factors have been shown to affect the development of the microbial populations on MAP meats. These selective pressures tend to be studied individually under laboratory conditions and extrapolation to the meat environment should be done with the utmost care. Consideration must be given to the synergistic action of these effects also. Indeed, the inhibitory action of CO_2 is greatly enhanced at low temperatures (Blickstad *et al.*, 1981). Other synergistic effects may also occur.

Lactic acid bacteria are "generally regarded as safe" (Church, 1993) and as such may have potential for use in the meat industry. Growth of particular strains may be encouraged or even inoculated onto the meat surface in order to produce "natural"

Table 7.2 Antimicrobial activity of diacetyl

	% of strains inhibited by diacetyl at the concentrations and pHs shown					
	pH 5.5			pH 6.0		
Organism	100μg ml^{-1}	200μg ml^{-1}	300μg ml^{-1}	100μg ml^{-1}	200μg ml^{-1}	300μg ml^{-1}
LAB	0	10	10	0	0	0
Gram +ve	0	63	75	0	36	91
Non LAB						
Yeast	0	100	100	25	100	100
Gram -ve	46	100	100	29	100	100

LAB - Lactic acid bacteria; Gram +ve, Gram positive; Gram -ve, Gram negative
Data from Jay (1982)

antimicrobial agents. This is particularly useful in a climate where consumer preferences are causing the food industry to reduce the quantities of additives and preservatives.

The factors affecting the growth and survival of the species described in this study need to be determined, such that conditions could be manipulated to allow selection of the required species. Use of LAB as "protective" cultures may alter the development of the microbial populations, this would need to be elucidated. The inoculation of LAB onto meat would alter the description from "fresh" meat to meat "product" according to current regulations. There are also problems regarding both inoculation and selection of particular species. The growth of LAB is enhanced whilst that of pseudomonads and *Brochothrix* diminished in elevated levels of CO_2 and reduced O_2. At present, such atmospheres are detrimental to meat colour and are unacceptable to consumers. A note of caution should be sounded, also, with regard to the potential of LAB to cause disease. Lactobacilli and leuconostocs have been found in human clinical

samples (Aguirre and Collins, 1993). In many cases the organisms were isolated in pure culture and were suspected to be opportunistic pathogens (Aguirre and Collins, 1993). Before LAB are used as biopreservative or probiotic in food systems, the risks should be fully evaluated.

Many species of LAB have been used for hundreds of years as starter cultures, particularly in the dairy industry and for the production of fermented meat products, apparently without causing disease. Lactic acid bacteria may be expected, therefore, to receive further study regarding their use in fresh meats. The results presented here indicate that *Lactobacillus sake/curvatus, Carnobacterium divergens*, or perhaps for meat stored in 100% CO_2, *Leuconostoc gelidum*, would be the organisms of choice for future research.

The initial microflora was found to have a profound effect on the species dominating during storage of MAP beef steaks. During the course of this study three trials were completed and detailed identification was done; results being different for each trial. It may be useful from a commercial viewpoint to determine the extent of, and factors causing, this variability. The shelf-life obtained with meat of different types and handling regimes could be determined. A model to assess the shelf-life may be obtained, therefore, given information on the microflora present initially and the methods of handling and packaging.

During the course of this study, particular species were found to be prevalent on MAP beef steaks, *viz. Pseudomonas fragi, Lactobacillus sake/curvatus, Carnobacterium divergens* and *Hafnia alvei*. The reason for their apparent competitive success is unknown. This would be an interesting avenue for further study. The factors affecting the growth of these species *vis-à-vis* those found to be less numerous on the meat could be determined using a modified meat juice medium in tissue culture plates (or equivalent) such that absorbance measurements can be taken. The growth curves could then be determined and the effects of pH and acidification (with acetic, lactic and hydrochloric acids) and antimicrobial agents (e.g. diacetyl, hydrogen sulphide etc.) in combination with different incubation conditions (atmospheres and temperatures) tested. Subsequent research could involve combinations of species to study the competitiveness of strains.

Interactions of microorganisms on the meat itself may, however, be very different to results obtained from cultures grown in a homogenous meat juice medium. Meat is of variable composition (as indicated in Chapter 1, see Figure 1.1 and Table 1.2) and many niches would be available for the growth of micro-organisms. The spacial arrangements of bacteria on the meat could be determined. There has been increased interest recently in the utilisation of *in situ* probes (e.g. identification of high G+C Gram positive organisms in activiated sludge - Roller *et al.* 1994). Specific pieces of DNA or RNA oligonucleotide are labelled with a fluorescent marker. Whole cell hybridisation using these probes allows the identification of individual cells. This technique may be adapted for use with bacterial cells on meat samples.

APPENDIX 1

Modified atmosphere packaging of red meats - previous studies

The packaging of red meats in modified atmospheres has been of increasing commercial importance during the last 20 years. Table A1.1 shows the findings of some of the previous studies on MAP red meats.

Table A1.1 The modified atmosphere packaging of fresh red meats

Meat		Storage characteristics				Spoilage characteristics				
Species	Muscle type	Temp.	Atmosphere	OTR[a] of film	Gas to Meat ratio	Shelf life[b] (days)	Cause of spoilage[c]	Dominant organisms[d]	Comments	Ref.
Beef	Round	1	60% CO_2 20% O_2 + 20% N_2 + aerobic	Drum	N.S.	>14	-	G -ves	Found residual	8
			20% CO_2 + 25% O_2 + 55% N_2 + aerobic			>14	-		effect	
			Air			<14	O			
		0	VP*							
				0		>105	O	LAB/B^{si}	Colour	13
				190		>105			changes	
				290		77-105			sooner but no	
				532		42-63			rejection	
				818		28-42				
				920		14-28				
	DFD	10	VP	300		5	OC	N.D.	Thought	12
			VP + glucose			5	C		*Alteromonas* -	
			VP + citrate			8	C		greening	
			VP + citrate + lactate			>14	-		*S liq* odour	
	Rump	0	100% N_2	0.4	N.S.	0	C	N.D.	Meat	1
			0.5% CO + N_2			24	O		inoculated with	
			1% CO + N_2			>30	-		104 of	
			10% CO + N_2			>30	-		*Moraxella* /	
		5	100% N_2			0	C		*Pseudomonas*	
			0.5% CO + N_2			20	O		mixture	
			1% CO + N_2			24	O			
			10% CO + N_2			>30	-			
		10	100% N_2			0	C			
			0.5% CO + N_2			8	O			
			1% CO + N_2			10	O			
			10% CO + N_2			12	O			

Strip loin	-1.5	CO_2	Foil lam.	1:1/2:1	>7/>7	-	N.D.	Residual	38
		CO_2 + 0.1% O_2			1/>7	C/-		oxygen	
		CO_2 + 0.2% O_2			<1/<7	C/C		level very	
High pH		CO_2			>7/>7	-/-		important	
		CO_2 + 0.1% O_2			>7/>7	-/-			
		CO_2 + 0.2% O_2			<7/<7	C/C			
	0-2	VP then retail at 4 °C	30		35 + 72h	O	LAB/Ps[s,i]		2
					42 + 72h	C+O	LAB[i]		
					49 + 72h				
					63 + 60h				
					70 + 36h				
Low pH	1	VP	30-40		70-84	F/O	LAB[i]		22
		VP	0		70-84	F			
		100% CO_2		0.4:1	105-126	F			
				1:1	126-147	F			
				2:1	126-147	F			
High pH		VP	30-40		<49	C/O		Ents	
		VP	0		<49	F		caused	
		100% CO_2		0.4:1	70-84	O		spoilage in	
				1:1	84-105	O		some cases	
				2:1	84-105	F			
	3	VP	<20		22-30	O			36
		100% CO_2		N.S.	22-30	O		*Ps. putida*	
		40%CO_2 + 60% N_2			22	O	Lb[i]	unusually	
		20% CO_2 + 80% O_2			7-14	O	Leu	common	
							Lb		
							Lb/Leu/Ps		
Ribeye rolls	-1	100% air	N.S.	N.S.	<23	C	Aerobic, not LAB[s]		3
		100% CO_2			<23				
		100% N_2			<23				
		100% O_2			<23				
		70% N_2 + 25% CO_2 + 5% O_2			<23				

Knuckles	1-3	High VP (25.8mmHg)	0.08-0.15		>28	-	Lb^{si}		6
		Medium VP (16.6mmHg)			14-21	O		Ents 12%	
		Low VP (11.2mmHg) Partial evacuation only			>28	-			
		Aerobic			>4	-	N.D.		
Roasts	1-3	VP	32		34	O	Lb^{si}		9,10
		100% O_2		1:1	13	O/C	Ps/Lb		
		20%CO_2 + 80% N_2			34	O/C	Lb		
		50% CO_2 + 50% O_2			27	O/C			
		20% CO_2 + 80% O_2			20	O/C			
		25% CO_2 + 25% O_2 + 50% N_2			20	O/C	Lb/Ps		
		51% CO_2 + 30% O_2 + 18% N_2 + 1% CO			34	O	Lb		
Steaks	1	75% O_2 + 25% CO_2	8.1	N.S.	13d	App	Leu^{i}		15, 16
		VP 14d then 75% O_2 + 25% CO_2			14+13	App			
		VP 28d then 75% O_2 + 25% CO_2			14+13	App			
	1	VP	40		70	N.D.	LAB^{s}	Ps. growth	33
	4				56			on film	
Hung 7d	1	75% O_2 + 25% CO_2	<2-4	2:1	21-25	O/C	Leu^{si}		23
VP 7d					21-28	O			
VP 21d					35	O/C			
Hung 7d	6				11-13	O	Lb/Ps/B/En		
VP 7d					11-13	O	t		
VP 21d					25	O	Leu		
	2	50% CO_2 + 15% O_2 + 35% N_2	<15.5	N.S.	0+8 retail	N.S.		Master	26
					7+8		$Ps/Lb/Ach^{si}$	pack	
					14+4				
					21+2				
High pH	0-2	VP	N.S.	N.S.	42	O	LAB/G-ve		32
Low pH	3	VP	2-4	N.S.	>45	-	LAB^{s}	Colour	29
		100% CO_2			>45	-		better with	
High pH		VP			34	O	LAB/Ent	storage in	
		100% CO_2			>45	-	LAB	CO_2	
Pieces	4	Air	10	10:1	14	O	B^{si}		17
Low pH		VP			>21	-	LAB		
		100% CO_2			>51	-			
		78% N_2 + 20% CO_2 + 2% O_2			21	O	B		

Pieces	4	Air			14	O			
High pH		VP			>21	-	LAB		
		100% CO_2			>51	-			
		78% N_2 + 20% CO_2 + 2% O_2			21	O	B		
Sirloin	1	75% O_2 + 25% CO_2	<10	2:1	18-22	O	LAB[s]		24
		VSP	<5		38	O			
Chucks									
Low pH	0-1	VP	N.D.		>66	-	LAB[si]		34
High pH					<66	O			
Loin	2	VP then retail in Air			24+6	N.D.	LAB[i]		35
Steaks		Medium O_2 barrier	N.D.		24+30				
		High O_2 barrier	N.D.		24+30		Lb		
Rounds	0	Air (retail)	Foil lam.	>10:1	N.S.	N.D.	LAB[i]	Used	31
		100% CO_2			15			anaerobic	
		VP			9			conditions	
	2	Air (retail)			N.S.			throughout	
		100% CO_2			15			for	
		VP			9			anaerobic	
	4	Air (retail)			NS			counts.	
		100% CO_2			15			Unusual	
		VP			9			numbers of staphs	
Cuts	1	VP	30-40	2:1	108	O/F	LAB	E.S.	20
Low pH			2		108	F	LAB		
			0		>108	-	LAB		
		CO_2	0		>108	-	Lb		
High pH		VP	30-40		66	O	Lb	High pH	
			2		87	O	Lb	higher Ents	
			0		87	O/F	Lb		
		CO_2	0		>108	-	Lb		
Trim/	1-2	Trim VP with solid CO_2, then mince and retail	40		30+7	-	N.D.	2g CO_2	21
Mince		display						absorbed in 24h Colour better	

	Ground Beef	2	50% CO_2 + 15% O_2 + 35% N_2	<15.5	N.S.	0+8 retail 7+6 14+2	N.S.	N.S.	Master Pack	26
Lamb	Chops	-1	Air	N.S.	N.S.	14	CO	B/Ps[si]		7
			80% Air + 20% CO_2			21	CF	B/Ps		
			80% O_2 + 20% N_2			21	CF	B/Ps		
			80% O_2 + 20% CO_2			21	CF	B		
			80% N_2 + 20% CO_2			42	C	B/Ps/Ent		
			80% H_2 + 20% CO_2 Low O_2			42	C	B/Ps/Ent		
			80% N_2 + 20% CO_2			56	F	B/Lb/Ent		
			80% H_2 + 20% CO_2 Oxygen free			56	F	B/Lb/Ent		
	Long loins	-1.5	CO_2	Foil lam.	1:1/2:1	>7/>7	-/-	N.D.		38
			CO_2 + 0.1%O_2			<7/>7	C/-			
			CO_2 + 0.2%O_2			<1/1	C/C			
Pork	Loins	4	100% Air	N.D.	2:1	3-7	O	Ps[si]		11
			100% CO_2			>35		LAB		
			100% N_2			approx 7		Ps		
	Roasts	1-3	VP	32	1:1	28	O	Lb[si]		4,5
			100% O_2			14		Ps		
			20%CO_2 + 80% N_2			21		Leu		
			50% CO_2 + 50% O_2			14		Leu		
			20% CO_2 + 80% O_2			14		Leu/Ps		
			25% CO_2 + 25% O_2 + 50% N_2			14-21		Leu		
			51% CO_2 + 30% O_2 + 18% N_2 + 1% CO			14	C	Leu		
		1	50% CO_2 + 25% O_2 + 25% N_2	Drum	>2:1	>14	C	N.D.	Residual effect	8
			50% CO_2 + 25% O_2 + 25% N_2 + aerobic retail			14+ 3-7				

Chops	0	Aerobic retail display at 0 °C			>7	-	Ps^{s}	Difficult to	37
		Aerobic retail display at 5 °C			5	App	LAB	assess data	
		VP + retail display at 0 °C	67		14+7	App	Ps		
		+ retail display at 5 °C			14+2-5	App	Ps		
		100% CO_2 + retail display at 0 °C		N.S.	14+>7	-	Ps		
		+ retail display at 5 °C			14+2-5	App	LAB/Ps		
	2	50% CO_2 + 15% O_2 + 35% N_2	<15.5	N.S.	0+8 retail	N.S.	Ps/Ent^{si}	Master	26
					7+8			Pack	
					14+6				
					21+2				
	4	Air	Imperm.	N.S.	2-3		Ps^{si}	Irradiation	25
		CO_2			8		LAB + B	treatment	
		N_2			8			12d	
		50% CO_2 + 50% N_2			8			Yeasts	
		25% CO_2 + 75% N_2			8			dominate	
		10% O_2 + 70% CO_2 + 20% N_2			8			flora in air	
	5	Aerobic retail display at 0 °C			5		LAB^{s}		37
		Aerobic retail display at 5 °C			5		LAB		
		VP + retail display at 0 °C	67		14+2-5		LAB/Ps		
		+ retail display at 5 °C			14+2-5		Ps/LAB/Ent		
		100% CO_2 + retail display at 0 °C		N.S.	14+>7		Ps/LAB		
		+ retail display at 5 °C			14+0-2		LAB/Ent		
	10	Air	N.S.	N.S.	N.S.		$LAB + B^{si}$		25
		CO_2			N.S.				
		N_2			N.S.				
		50% CO_2 + 50% N_2			N.S.				
		25% CO_2 + 75% N_2			N.S.				
		10% O2 + 70% CO_2 + 20% N_2			N.S.				
Steaks	-1	40% CO_2 + 60% N_2		5:1	56	C/O	B^{s}	Aseptic vs	27
	4.4				35	C/O	LAB	commercial	
	10				14-21	O	Ent	meat	
	2	20% CO_2 + 80% air	25	>2:1	16-21	O	B^{si}		28
		20% CO_2 + 80% O_2			16-21	O	B		

Long loins	-1.5	CO_2	Foil lam.	1:1	>7	-	N.D.	38
		CO_2 + 1.0%O_2			<7	C		
		CO_2		2:1	>7	-		
		CO_2 + 1.0%O_2			>7	-		
Loins	0	VP	8	N.S.	15	App	LAB^{si}	19
		100% Air	10	2:1	15-20	O	Ps/B^{si}	18
		100% CO_2			79-119	O	LAB	18
	1	75% O_2 + 25%CO_2	<10	2:1	10-14	O	LAB/B^{s}	24
		VSP	<5		20	O	LAB	24
	3	VP	8	N.S.	8	App	LAB^{si}	19
	4	100% Air	10	2:1	8-12	O	Ps^{si}	18
		100% CO_2			27-40	O	LAB	18
	4	100% Air	Foil lam.	>2:1	<11	O	Ps^{si}	14
		1 atm CO_2			41	O	LAB	14
		5 atm CO_2			>121	-	LAB	14
	7	VP	8	N.S.	8	App	LAB^{si}	19
	14	100% Air	Foil lam.	>2:1	3	O	Ps^{si}	14
		1 atm CO_2			7	O	LAB	14
		5 atm CO_2			>15	-	LAB	14
Loins then retail chops	-1.5	CO_2 (CAPTECH) then retail overwrap	Foil lam.	2.5:1	168 84+4 168+3	- C/App C/App	LAB^{s}	30

a OTR = Oxygen transmission rate. Measured in ml m^{-2} 24 h^{-1} at 1 atmosphere. The temperature and relative humidity at the time of measurement varies with film data. Drum - Atmosphere created in a drum. Imperm. impermeable film. Foil lam. = foil laminate - theoretically impermeable.

b Where detailed the time is taken from the time of slaughter. The methods and times of aging differ. Where retail shelf-life was tested, time is given as time in modifed atmosphere + time in retail display.

c - No spoilage noted, O Off-odour, C Discolouration, F Off-flavour, App. General appearance.

d G-ve Gram negative organisms, LAB Lactic acid bacteria, B *Brochothrix thermosphacta*, Ps. *Pseudomonas* spp., Leu *Leuconostoc* spp., Ent Enterobacteriaceae, Lb *Lactobacillus* spp., Ach *Achromobacter* spp. S liq *Serratia liquefaciens*. Tested with ^{s} selective medium only, ^{i} identification of isolates from total counts or ^{si} selective media were used but isolates were also taken from total counts and identified.

* VP Vacuum pack

N.S. Not specified

N.D. Not determined

E.S. Electrical stimulation

References:

1 Clark *et al.* (1976)
2 Sutherland *et al.* (1975)
3 Huffman *et al.* (1975)
4 Seideman *et al.* (1979b)
5 Christopher *et al.* (1979b)
6 Seideman *et al.* (1976)
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9 Christopher *et al.* (1979a)
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APPENDIX 2

Identification of lactic acid bacteria from a variety of selective and elective media incubated anaerobically

INTRODUCTION

The characterisation of isolates was done using mainly simple carbohydrate tests, with clustering by numerical taxonomy techniques and identification by comparison with type strains incuded in the study and with results from previous studies (Table 4.3).

MATERIALS AND METHODS

The lactic acid bacteria were enumerated on a variety of elective and selective media as described in Chapter 2 (pp. 45). Representatives were randomly isolated, purified, maintained, characterised and identified as described in Chapter 4 (pp. 92-96). In outline, isolates were characterised using traditional carbohydrate and biochemical tests in a labour-saving microtitre-plate method where possible. The results from the characterisation were scored as positive (1) or negative (0) and analysed with numerical taxonomy using PHYLIP (Phylogeny Inference Package) programmes (Joseph Felsenstein, Dept. Of Genetics SK-50, University of Washington, Seattle, WA 98195, USA). The similarity between organisms was calculated using Jaccard (S_j) and simple matching (S_{SM}) co-efficients and clusters formed using UPGMA (Unweighted Pair Group Method).

RESULTS

Lactic acid bacteria were enumerated on a variety of selective and elective media. The numbers of bacteria present on the media have been presented previously (Chapter 2, pp. 45-50).

Both the Jaccard (Figure A2.1) and the simple matching co-efficient (Figure A2.2) were used to cluster the strains included in this study. The Jaccard co-efficient (S_j) showed some groupings similar to those of the simple matching co-efficient (S_{SM}). The percentage similarity of the clusters was lower in S_j than in the S_{SM} and the type strains did not necessarily cluster amongst the same organisms in the two studies. This has been noted previously (Borch and Molin, 1988). Clusters are evident (Figure A2.1 - S_j) even though

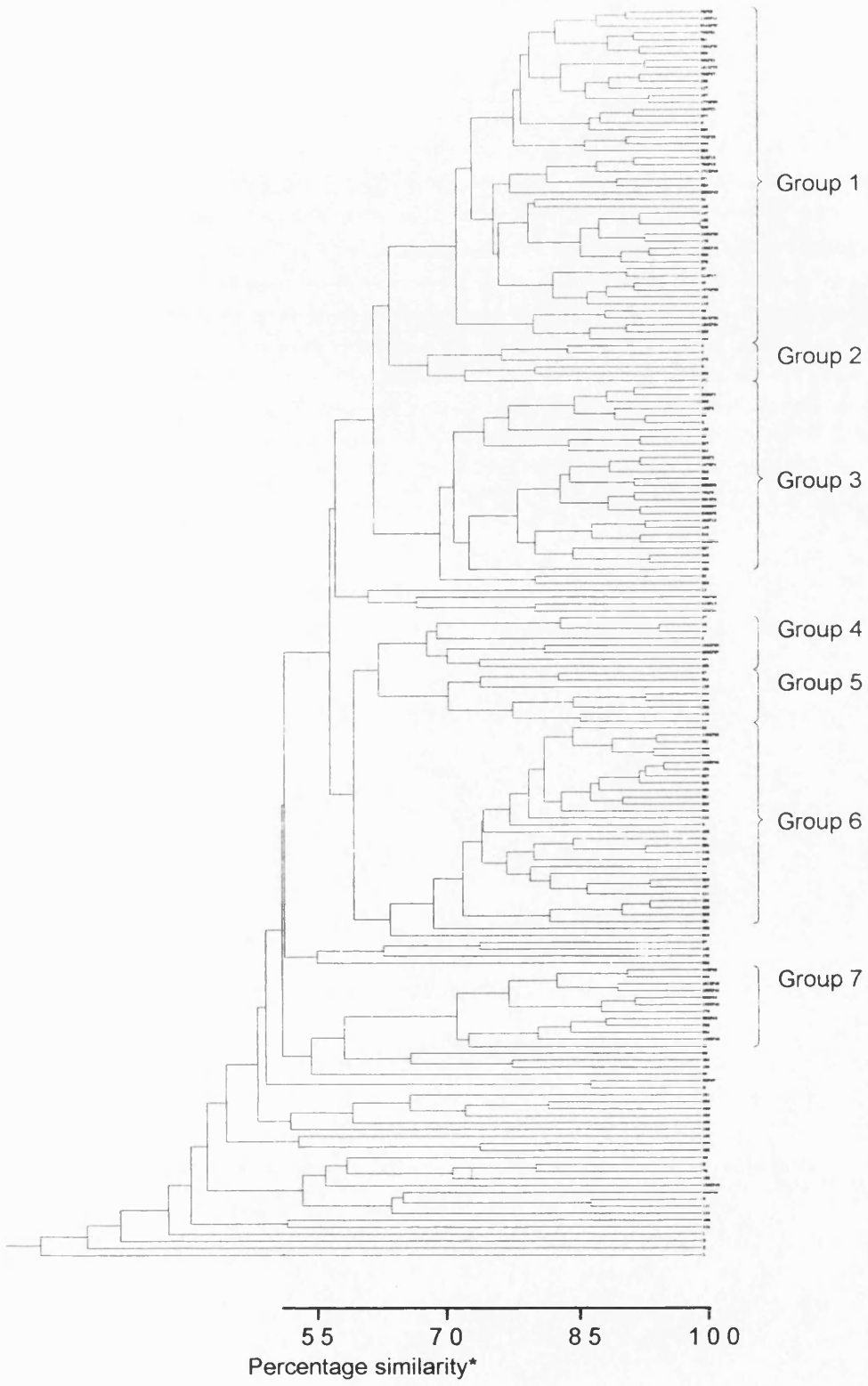


Figure A2.1 **Lactic acid bacteria isolated from a variety of elective and selective media, characterised by traditional methods and clustered by the Jaccard co-efficient**

*The scale is approximate, based on the percentage similarity calculated from the similarity co-efficients between pairs of strains. The calculation is an arithmetic mean and exact numbers cannot therefore be specified.

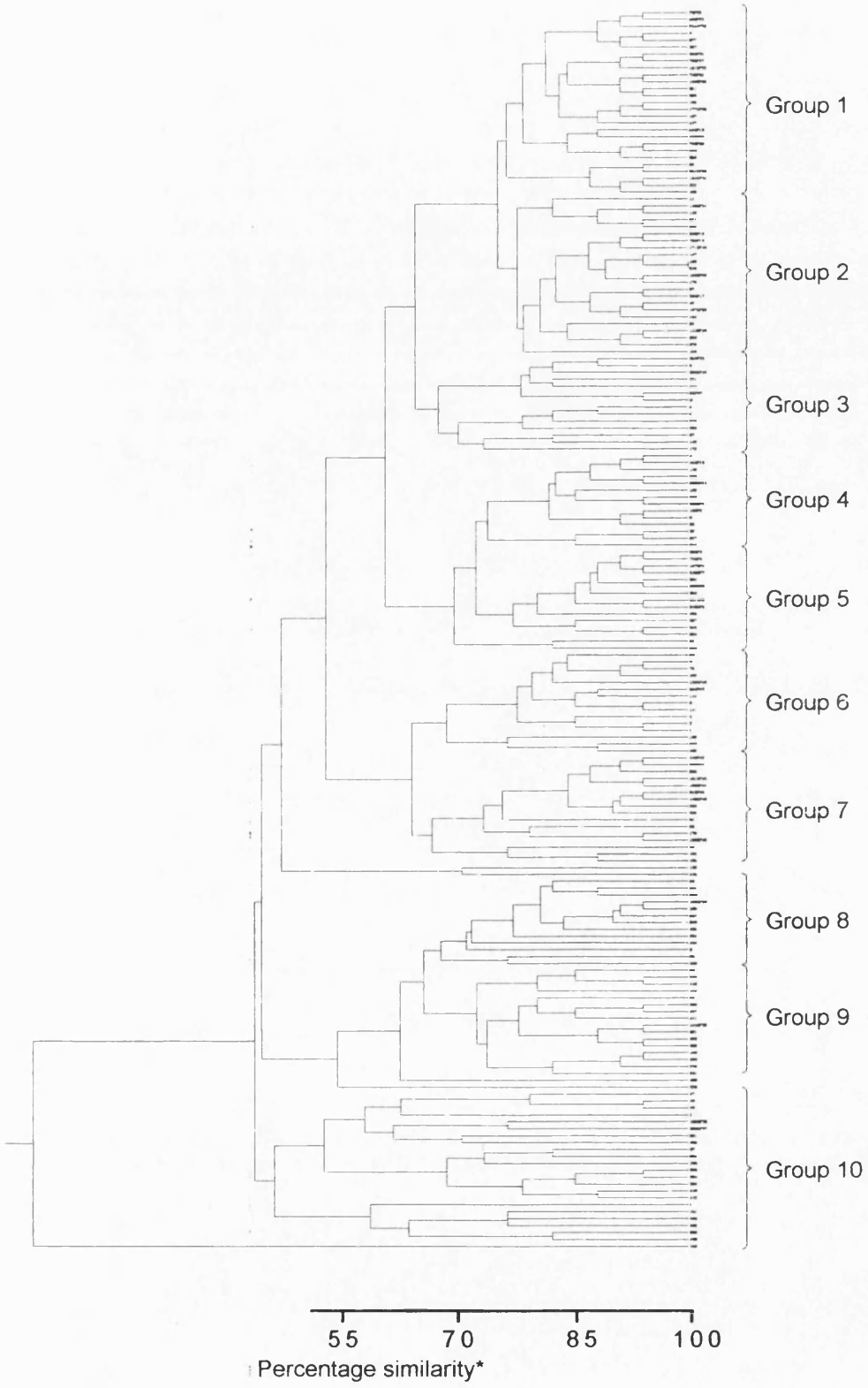


Figure A2.2 **Lactic acid bacteria isolated from a variety of elective and selective media, characterised by traditional methods and clustered by the simple matching co-efficient**

*The scale is approximate, based on the percentage similarity calculated from the similarity co-efficients between pairs of strains. The calculation is an arithmetic mean and exact numbers cannot therefore be specified.

many had <75% similarity; 70 - 80% is often taken as the approximate similarity for description of a species. This contrasts with the findings from the analysis using the simple matching co-efficient (Figure A2.2). Ten groups could be described with similarities of >70%. Indeed, groups 1 and 2, 4 and 5, and 6 and 7 could be regarded as three rather than six groups at this level of similarity; this may indicate the presence of biovars or subspecies. Although group 10 was evident with clustering, the percentage similarity was low (approximately 50%). It is probable that this group included strains which grew weakly on a particular medium. As noted above, the simple matching co-efficient was used to identify the lactic acid bacteria.

Comparison of the profiles of the different groups obtained in this study (Table A2.1) with that of others (Table 4.3) led to the tentative identification of the following:

Groups 1 and 2

One hundred and 94 strains were present in these groups as well as the type strain of *Lactobacillus sake* and another strain of the same species which was identified elsewhere (*Lactobacillus sake).* It was assumed, therefore, that these groups contained isolates which identified with *Lact. sake.* The occurrence of two groups probably indicates the presence of different sub-species or biotypes, or possibly isolates of *Lact. curvatus,* a very closely related organism the type strain of which occurred in group 3.

Group 3

This group, which was based on many variable (16-84%) results (Table A2.1) included *Lactobacillus curvatus, Pediococcus pentosaceus, Leuconostoc gelidum* and two strains of *Lactococcus lactis,* together with sixteen test strains. It is probable that the last mentioned were *Lact. curvatus.* It is evident from Figure A2.2 that group 3 is more closely aligned with groups 1 and 2 than with the other groupings. For the purposes of further analyses, however, the meat isolates will be deemed to be "unidentified".

Groups 4 and 5

These groups contained 63 meat isolates which were identified with *Carnobacterium divergens.* The occurrence of two clusters may well represent two biotypes.

		Tests used for the differentiation of lactic acid bacteria																					
Group	Total	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	84	$+^{93}$	$-^{0}$	$-^{0}$	v^{51}	$-^{8}$	$-^{8}$	$+^{100}$	$-^{2}$	v^{37}	$+^{100}$	$-^{0}$	$+^{89}$	$-^{8}$	$-^{0}$	$+^{100}$	v^{49}	$-^{0}$	$+^{92}$	$+^{99}$	$-^{0}$	$-^{1}$	$+^{88}$
2	112	$+^{95}$	$-^{2}$	$-^{11}$	v^{24}	$-^{1}$	$+^{99}$	$+^{98}$	$-^{0}$	$-^{0}$	$+^{100}$	$-^{9}$	v^{81}	$-^{0}$	$-^{0}$	$+^{99}$	$+^{94}$	$-^{0}$	$+^{100}$	$+^{94}$	$-^{0}$	$-^{1}$	$+^{91}$
3	21	v^{67}	$-^{14}$	$-^{0}$	$-^{5}$	$-^{0}$	v^{48}	v^{71}	$-^{0}$	v^{62}	$+^{95}$	$-^{10}$	$-^{0}$	$-^{0}$	$-^{0}$	$+^{95}$	v^{76}	$-^{0}$	v^{24}	v^{71}	$-^{0}$	$-^{0}$	v^{29}
4	20	$+^{100}$	$-^{10}$	$+^{100}$	$-^{15}$	$+^{100}$	$+^{95}$	v^{50}	$-^{0}$	v^{85}	$+^{100}$	$-^{0}$	$-^{5}$	$-^{5}$	$-^{0}$	$+^{100}$	$+^{100}$	$-^{0}$	v^{80}	v^{35}	$-^{0}$	$-^{0}$	v^{30}
5	44	$+^{95}$	$-^{5}$	$+^{93}$	v^{59}	v^{84}	$+^{95}$	$+^{91}$	v^{68}	$-^{9}$	$+^{95}$	$-^{0}$	$-^{0}$	$-^{0}$	$-^{0}$	$+^{100}$	$+^{93}$	$-^{0}$	$-^{2}$	$+^{98}$	$-^{0}$	$-^{9}$	v^{41}
6	16	v^{50}	v^{44}	$-^{6}$	v^{19}	$-^{0}$	$-^{0}$	$-^{0}$	$-^{0}$	$-^{0}$	v^{31}	$-^{0}$	$-^{0}$	$-^{0}$	$-^{0}$	v^{50}	$-^{0}$	$-^{0}$	v^{63}	v^{19}	$-^{0}$	$-^{6}$	v^{69}
7	26	$-^{4}$	$+^{92}$	$-^{4}$	$-^{8}$	$-^{0}$	v^{46}	$-^{0}$	$-^{0}$	$-^{4}$	$+^{92}$	$-^{0}$	$-^{4}$	v^{62}	$-^{4}$	$+^{96}$	$-^{12}$	$-^{0}$	$+^{96}$	$+^{96}$	v^{46}	$-^{8}$	$+^{88}$
8	14	v^{21}	$+^{100}$	v^{79}	$+^{93}$	v^{71}	$+^{100}$	$+^{93}$	$-^{0}$	v^{50}	$+^{93}$	$-^{7}$	$+^{93}$	v^{54}	$+^{100}$	$+^{100}$	v^{79}	$-^{0}$	$+^{100}$	$+^{93}$	v^{79}	$+^{100}$	$+^{93}$
9	16	v^{19}	$+^{94}$	v^{31}	$+^{94}$	v^{56}	$+^{100}$	$-^{0}$	$-^{0}$	v^{69}	$+^{100}$	$-^{0}$	v^{75}	$-^{0}$	v^{81}	$+^{100}$	v^{81}	$-^{0}$	$+^{100}$	$+^{100}$	$-^{13}$	$+^{100}$	$+^{88}$
10	29	v^{59}	v^{17}	v^{76}	v^{48}	v^{69}	v^{69}	v^{55}	v^{49}	v^{66}	v^{72}	v^{59}	v^{72}	v^{14}	v^{59}	v^{38}	$+^{100}$	$-^{7}$	v^{79}	v^{79}	v^{17}	v^{17}	v^{21}

Groups: Group 1 and 2, *Lactobacillus sake/curvatus*; Group 3, Unidentified; Group 4 and 5, *Carnobacterium divergens*; 6 and 7, *Leuconostoc carnosum*; Group 8, *Leuconostoc gelidum; Group 9. Leuconostoc mesenteroides*; Group 10, Unidentified

Tests: 1, Cell morphology (rod = +, coccus = -); 2, Production of CO_2 from glucose; Fermentation of: 3) amygdalin; 4) arabinose; 5) arbutin; 6) cellobiose; 7) galactose; 8) lactose; 9) maltose; 10) mannose; 11) mannitol; 12) melibiose; 13) methyl-D-glucoside; 14) raffinose; 15) ribose; 16) salicin; 17) sorbitol; 18) sucrose; 19) trehalose; 20) turanose; 21) xylose; 22) Production of hydrogen peroxide

\+ 85-100%

\- 0-15%

v 16-84%

Table A2.1 Characteristics of each group of lactic acid bacteria, calculated from the simple matching co-efficient.

Groups 6 and 7

Thirty seven meat isolates clustered in these two groups which encompassed five type strains, *Ped. acidilactici, Ped. pentosaceus, Lact. fructivorans, Lactococcus pentosaceus* and *Carnobacterium mobile.* As the profiles of both these groups were similar to that of *Leuconostoc carnosum* (Table 4.3), the meat isolates were tentatively identified with this species.

Group 8

Only thirteen field strains occurred in this cluster. As the profile matched that of *Leuconostoc gelidum,* they were tentatively identified with this species.

Group 9

The profile of *Leuconostoc mesenteroides* (Table 4.3) matched closely that of organisms in group 9. As such these 16 field strains wre identified with the named organism.

Group 10

The remaining strains did not cluster to a significant level. These 29 isolates, including *Lact. plantarum* and *Lact. bavaricus,* were left assigned to a particular taxonomic status and will be referred to as unidentified.

APPENDIX 3

Identification of lactic acid bacteria from meat acidfied with acetic acid

INTRODUCTION

This experiment was done because of the current use of acid rinses and sprays for carcass decontamination. As acetic acid is used in many such sprays, there is a distinct possibility that it may select lactic acid bacteria. The experiment involved the deliberate acidification of minced beef with storage in vacuum packs at chill temperatures. The microbial populations occurring throughout storage were elucidated using a variety of selective media. Lactic acid bacteria were isolated at time zero and during the first two weeks of storage in order to determine the species surviving the acidified conditions.

MATERIALS AND METHODS

Minced rump of beef was purchased from a local butcher. Four hundred and 50 g of meat were acidified with 14.4 ml of 50% (v/v) filter-sterilised acetic acid. The acid was added slowly and thoroughly mixed between additions. Using the same method, a further 450 g were acidified with 7.2 ml acietic acid and 7.2 ml distilled water. With control meat samples the 450 g had 14.4 ml distilled water added. The final pH of each preparation was determined (average of five readings) using a Pye Unicam pH metre. Table A3.1 lists the pH of the meat as well as the calculated proportions of undissociated acid present.

Table A3.1 Addition of acetic acid to minced beef rump

Sample	Meat weight (kg)	Amount of liquid addition (ml)		pH	% Undissociated acid	
		Water	50% acetic acid		at particular pH	per 100g meat
Control	0.45	14.4	0	5.68	-	-
Acidifed	0.45	7.2	7.2	4.88	41	0.33
Highly acidified	0.45	0	14.4	4.45	67	1.07

One hundred g samples were put in VP pouches (Green Crown Packaging Ltd; Oxygen transmission rate 43 cm^3 m^{-2} d^{-1} at 23 °C and 75% relative humidity) and vacuum

packed. The remainder of the meat was analysed for extract release volume (Jay, 1964). Twenty five g was macerated for 2 minutes with 100 ml of cold (*ca* 4 °C) water. The homogenate was filtered through Whatman filter paper No. 1 for 15 min. The volume of exudate was determined in a measuring cylinder.

The packs were stored at 5 °C and sampled at regular intervals. The surface of the pack was wiped with 70% (v/v) ethanol to eliminate contamination. Ten g were homogenised with 90 ml MRD (Lab M) for 60 s in a Colworth Stomacher. A decimal dilution series was made and appropriate dilutions plated onto the media shown in Table A3.2. In cases where low numbers of bacteria were expected, 10 g of the acidified meat were homogenised in 20 ml of MRD before plating.

Table A3.2 Media used to determine the microbial populations developing on acidified minced beef rump

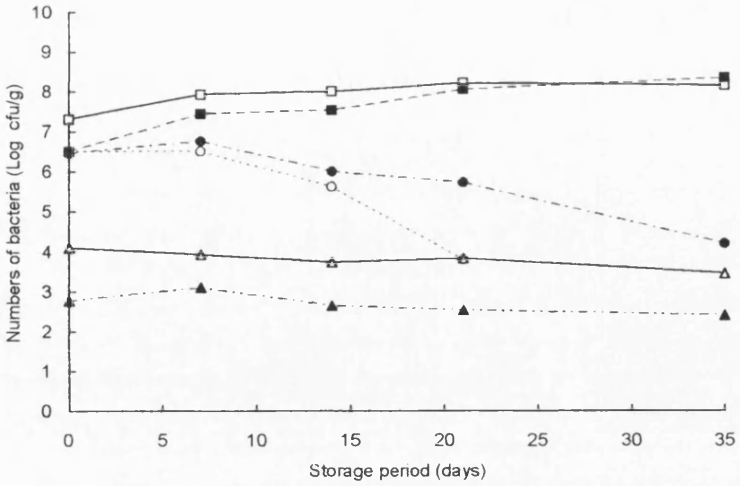
Medium[a]	Bacteria selected	Inoculation Method[b]	Incubation conditions: Atmosphere[c]	Temp. (°C)	Time (h)
APT	Total aerobic count	Spread	Aerobic	25	72
MRS	LAB	Pour	Anaerobic	25	72
CFC	*Pseudomonas*	Spread	Aerobic	25	48
STAA	*Brochothrix*	Spread	Aerobic	25	48
RBC	Yeast	Spread	Aerobic	25	120
VRBG	Enterobacteriaceae	Pour	Aerobic	30	48

a APT, All Purpose Tween (Difco); MRS, de Man, Rogosa, Sharp (Lab M); CFC, Cephaloridine fucidin cetrimide (Lab M); STAA, Streptomycin thallous acetate actidione agar (Lab M); RBC, Rose Bengal Chloramphenicol (Lab M); VRBG, Violet red bile glucose agar (Lab M)
b 100 µl of the appropriate dilutions were spread onto plates, 1 ml being used for pour plates
c Anaerobic gas generating system (Oxoid) was used to create anaerobic conditions

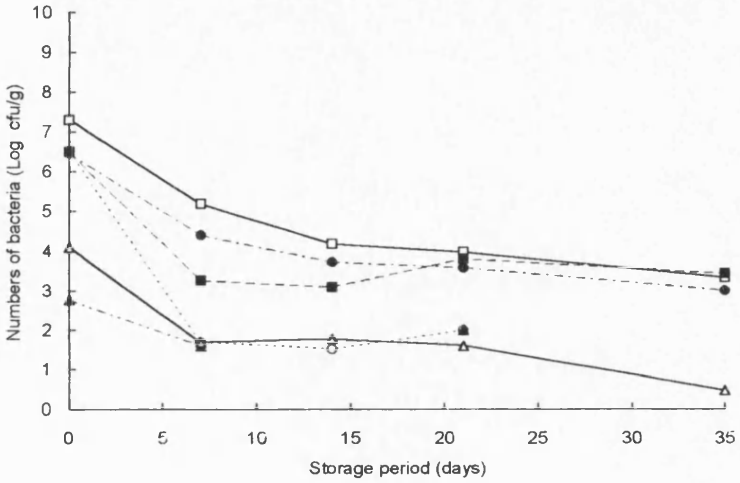
Lactic acid bacteria were isolated (from MRS) from the three meat preparations during the first two weeks of storage. These were purified, characterised and identified using the methods described in Chapter 4 (pp. 92-96).

RESULTS

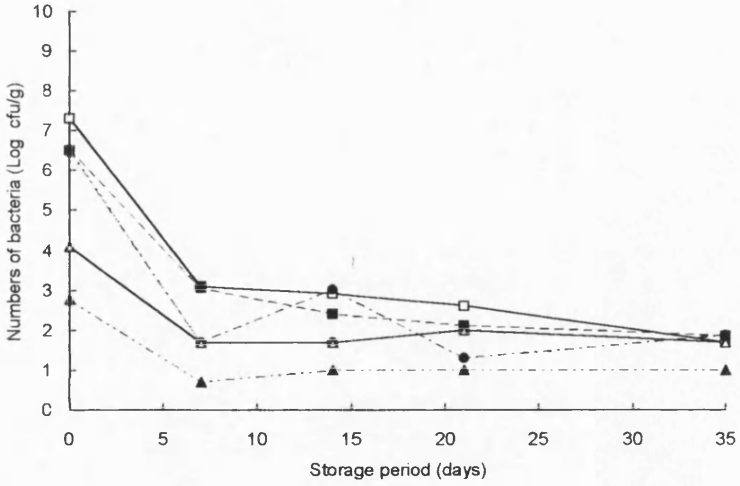
The results for the microbial enumeration in the acetic acid experiment are shown in Figure A3.1. Initially, there were large numbers of bacteria on the minced beef rump. Lactic acid bacteria, *Brochothrix thermosphacta* and pseudomonads were the predominant organisms at the outset. During storage of the vacuum packed control (water addition) meat, however, the numbers of *Pseudomonas* spp. and particularly *Broch.*



a) Control



b) Acidified



c) Highly acidified

Figure A3.1 **Microbial flora developing on vacuum packed minced rump**
□ Total aerobic count (APT); ■ Lactic acid bacteria (MRS); ○ *Brochothrix thermosphacta* (STAA); ● *Pseudomonas* spp. (CFC); ▲ Enterobacteriaceae (VRBG); Δ Yeast (RBC).
Enterobacteriaceae below the limit of detection (10^2 cfu g^{-1}) on the acidified meat on day 14 and 35. *Brochothrix* below limit of detection day 35 on the acidified meat and days 21 and 35 on the highly acidified meat.

thermosphacta declined, whilst the numbers of lactic acid bacteria increased. Yeasts, present at the beginning of storage at approximately 10^4 g^{-1}, maintained this level throughout. A similar situation obtained with the Enterobacteriaceae which occurred at approximately 10^3 g^{-1} throughout the storage of the meat.

The numbers of all groups of bacteria declined during storage of meat acidified with the lower concentration of acetic acid (Figure A3.1b). The largest decrease in numbers occurred in the first week of storage, particularly with *Broch. thermosphacta*, the most acid-sensitive organism. Somewhat surprisingly, the pseudomonads appeared to be least affected by the acetic acid. The number of the lactic acid bacteria increased during the third week of storage.

Figure A3.1c shows the effect of the higher concentration of acetic acid on the micro-organisms. As with the lower acetic acid addition, all the numbers of bacterial groups decreased during storage. During the first week at 5 °C, the total number of aerobic bacteria (as determined on APT) fell to *ca* $\log_{10}$ 3.1 $cfug^{-1}$. By the end of the storage period the microbial count fell to $\log_{10}$ 1.7). The changes in the numbers of micro-organisms in highly acidified meat was similar to those of the acidified meat. *Brochothrix* numbers fell to log 1.7 cfu g^{-1} during to the first week of storage; this population size persisted throughout the remainder of the storage period.

The pH changes during storage of the acidified and control meats are shown in Figure A3.2. The pH of the acidified meats was not significantly different at the end to that at the beginning of storage, although the pH of both treatments increased during the second week of storage before returning to the initial pH by day 35. The control meat, however, had an initial pH of 5.68 which decreased to pH 5.11 by the end of storage.

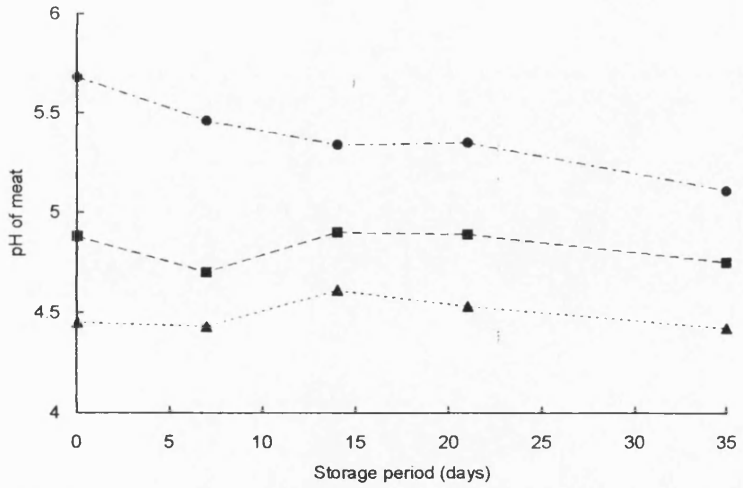


Figure A3.2 pH during storage of vacuum packed meat with addition of acetic acid or water

●, Control (water addition); ▲, Acidified, ■, Highly acidified meat

Lactic acid bacteria were isolated (from MRS plates) from the three meat preparations stored for up to 2 weeks at 5 °C. These were characterised and subsequently clustered using both the Jaccard (Figure A3.3) and simple matching (Figure A3.4) co-efficients.

As observed with the previous cluster analysis (pp. 97-100 and 184-187), the Jaccard co-efficient (S_j) revealed largely similar groupings to those formed using the simple matching co-efficient (S_{SM}). There were, however, differences between the two analyses with respect to the location of the type strains. The percentage similarity in the Jaccard analysis tended to be less than with the simple matching, as noted previously. *Carnobacterium mobile* was located in a group with *Carn. divergens* in the S_j clustering, but along with *Lact. curvatus* in that of the S_{SM}.

With the simple matching co-efficient, four groups were observed (characteristics shown in Table A3.3).

Group 1

Twenty five meat isolates and strains of *Lact. sake* (2 strains), *Lact. curvatus* and *Carn. mobile* clustered together in group 1. As the two *Lactobacillus* spp. are difficult to distinguish, the isolates in this group were identified with *Lact. sake/curvatus.* There were two sub-groups. The first contained 5 test strains and the two previously identified *Lact. sake* strains, whilst the other contained the *Lact. curvatus* and *Carn. mobile* strains together with 18 meat isolates.

Group 2

This group contained the type strain of *Carn. divergens* and 11 strains isolated in this study. The test strains were assigned to *Carn. divergens.*

Group 3

Sixteen meat isolates clustered with *Leuconostoc mesenteroides* and *Leuc. gelidum* type strains. The isolates were identified with the genus *Leuconostoc,* but identification to species level was not possible with the adopted methods as the profiles of the test strains (Table A3.3) did not match exactly those of either species (Table 4.3).

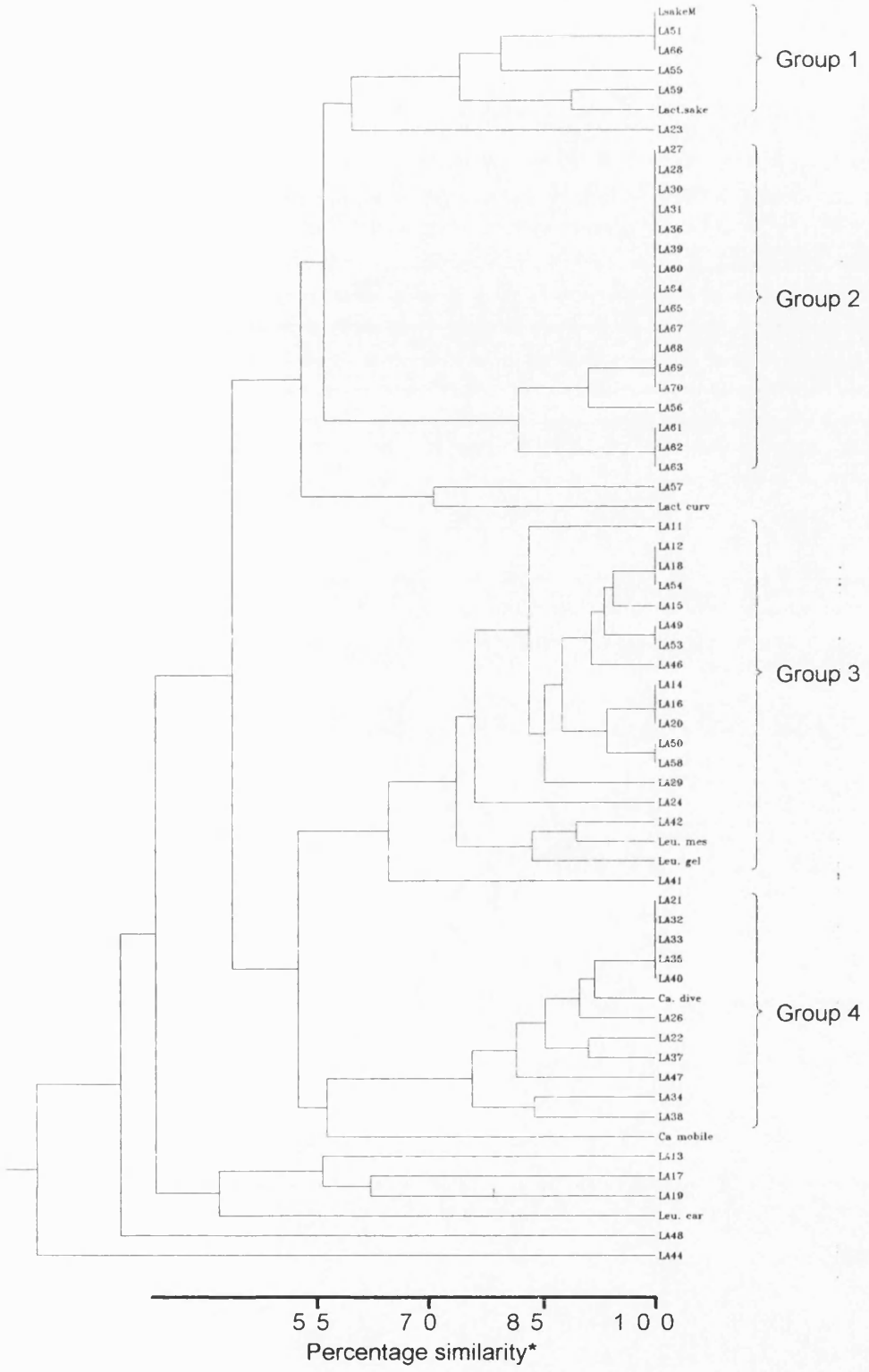


Figure A3.3 **Lactic acid bacteria isolated from meat acidified with acetic acid or with water addition, characterised by traditional methods and clustered with the Jaccard coefficient**

*The scale is approximate, based on the percentage similarity calculated from the similarity coefficients between pairs of strains. The calculation is an arithmetic mean and exact numbers cannot therefore be specified.

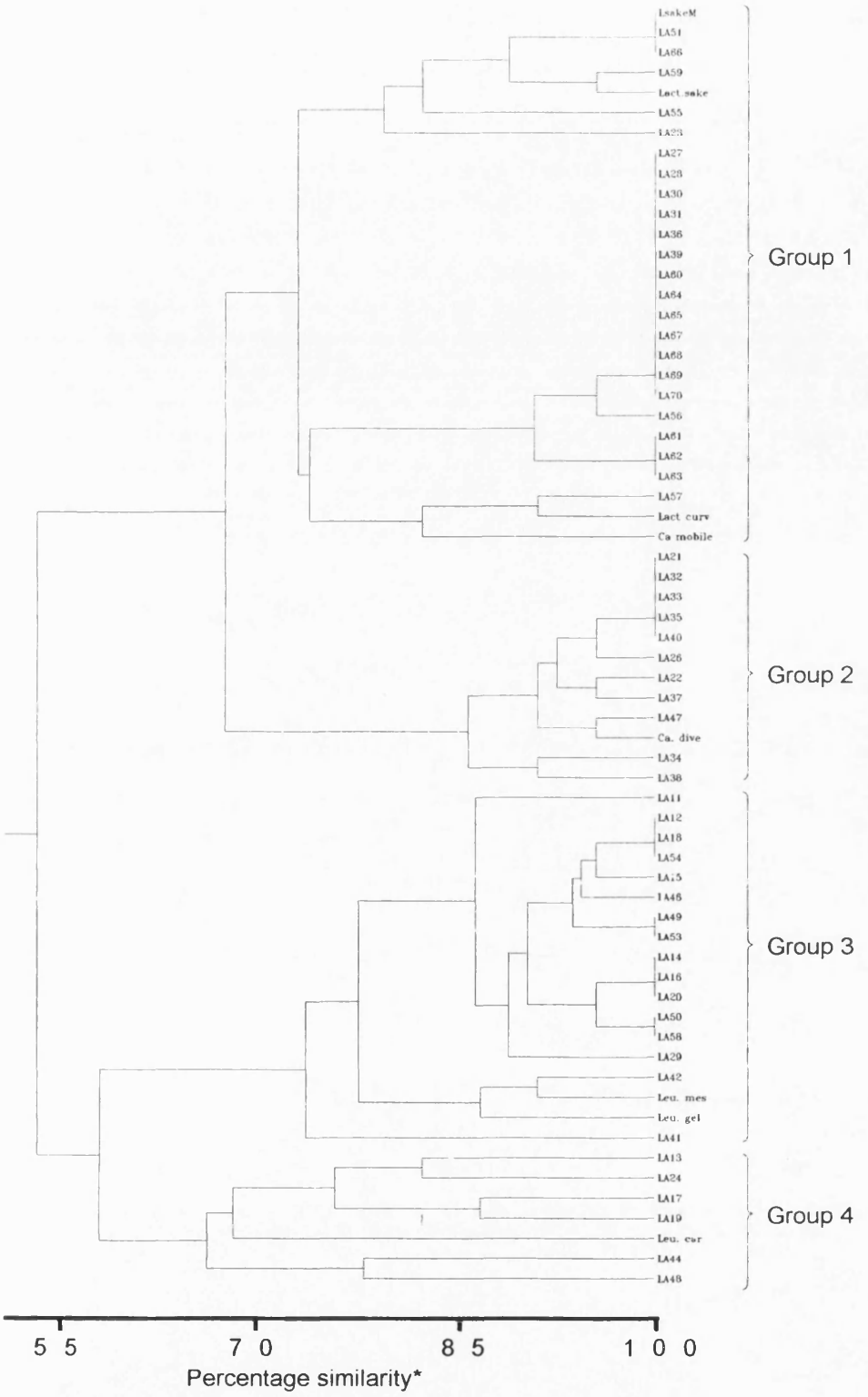


Figure A3.4 **Lactic acid bacteria isolated from meat acidified with acetic acid or unacidified, characterised by traditional methods and clustered with the simple matching coefficient**

*The scale is approximate, based on the percentage similarity calculated from the similarity coefficients between pairs of strains. The calculation is an arithmetic mean and exact numbers cannot therefore be specified.

		Tests used for the differentiation of lactic acid bacteria																					
Group	Total	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	27	$+^{96}$	$-^{4}$	$-^{4}$	$+^{81}$	$-^{4}$	v^{19}	$+^{96}$	v^{63}	v^{70}	$+^{100}$	$-^{0}$	v^{22}	$-^{0}$	$-^{0}$	$+^{96}$	v^{67}	$-^{0}$	v^{30}	$+^{89}$	$-^{0}$	$-^{0}$	v^{22}
2	12	$+^{100}$	v^{17}	$+^{100}$	$-^{0}$	v^{83}	$+^{100}$	v^{17}	$-^{8}$	$+^{100}$	$+^{100}$	$-^{0}$	$-^{0}$	$-^{0}$	$-^{0}$	$+^{92}$	$+^{100}$	$-^{8}$	$+^{100}$	$+^{67}$	$-^{0}$	$-^{0}$	$-^{0}$
3	18	$-^{6}$	$+^{100}$	$+^{89}$	$+^{89}$	$+^{78}$	$+^{100}$	v^{22}	$-^{0}$	v^{67}	v^{83}	$-^{0}$	$+^{94}$	v^{28}	$+^{100}$	$+^{100}$	$+^{100}$	$-^{6}$	$+^{100}$	$+^{94}$	v^{67}	$+^{100}$	$-^{0}$
4	7	$-^{0}$	$+^{100}$	v^{29}	v^{71}	$-^{0}$	$-^{0}$	$-^{0}$	$-^{0}$	v^{29}	v^{43}	$-^{14}$	$-^{14}$	$-^{14}$	v^{43}	v^{57}	$-^{14}$	$-^{0}$	v^{71}	v^{29}	v^{57}	v^{43}	$-^{14}$

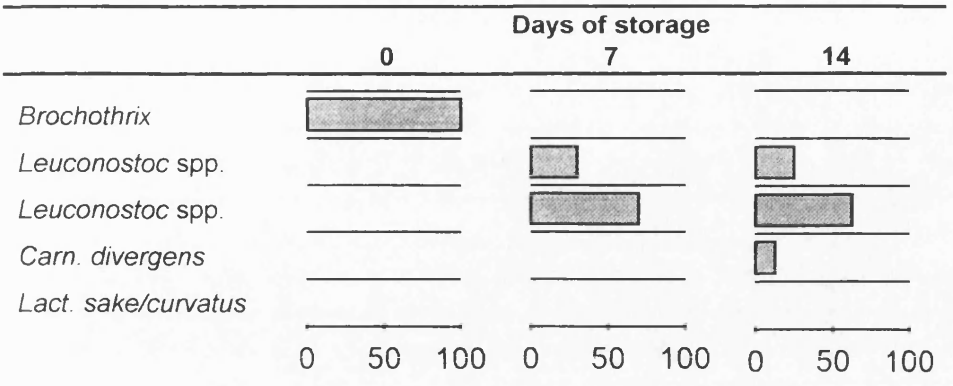
Groups: Group 1, *Lactobacillus sake/curvatus*; Group 2, *Carnobacterium divergens*; Group 3, *Leuconostoc* spp., Group 4, *Leuconostoc* spp.
Tests: 1, Cell morphology (rod = +, coccus = -); 2, Production of CO_2 from glucose; Fermentation of: 3) amygdalin; 4) arabinose; 5) arbutin; 6) cellobiose; 7) galactose; 8) lactose; 9) maltose; 10) mannose; 11) mannitol; 12) melibiose; 13) methyl-D-glucoside; 14) raffinose; 15) ribose; 16) salicin; 17) sorbitol; 18) sucrose; 19) trehalose; 20) turanose; 21) xylose; 22) Production of hydrogen peroxide
\+ 85-100%
\- 0-15%
v 16-84%

Table A3.3 Characteristics of each group of lactic acid bacteria from vacuum packed rump beef acidified with acetic acid, calculated from the simple matching co-efficient.

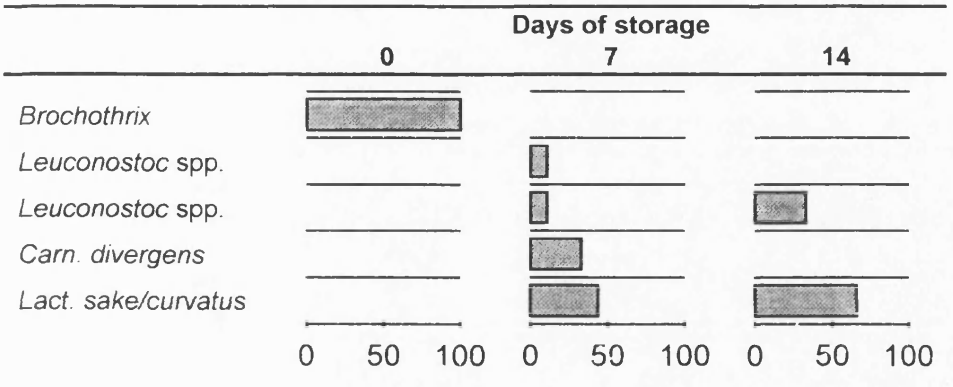
Group 4

Six meat isolates and the type strain of *Leuc. carnosum* grouped together. The similarity between the isolates was not as high as with the other groups. Many of the test strains gave variable (16-84%) results in the characterisation. This may indicate a heterogeneity amongst the group representatives. All strains were cocci, however, and could therefore be assumed to be leuconostocs. Further identification (to species level) was not attempted.

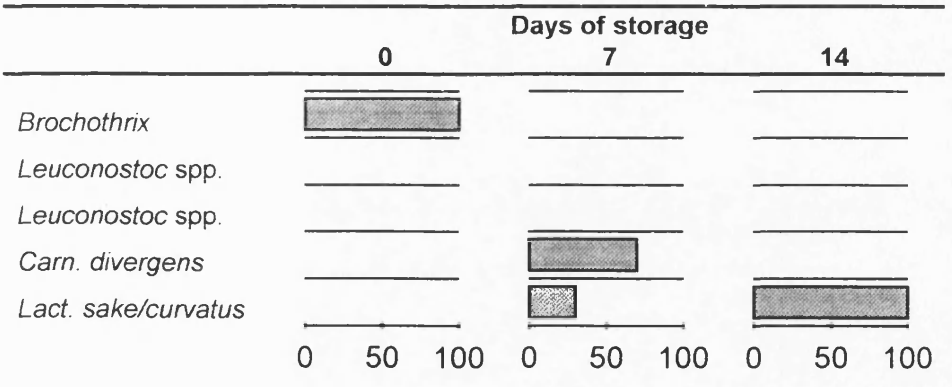
The succession of organisms developing on acidified vacuum packed beef mince are detailed in Figure A3.5. At the outset all of the micro-organisms isolated from MRS medium were *Brochothrix thermosphacta.* The subsequent development of microflora was dependent on the treatment of the meat. The control meat (water addition) contained a high proportion of *Leuconostoc* (> 85%) as compared to the acidified meats (< 35%). In numerical terms, *Carn. divergens* also appeared to be of increasing importance towards the end of the 14 day experiment with meat containing additional water only. A different profile was seen with the slightly acidified meat (Figure A3.5b). In this case, a mixture of lactobacilli, carnobacteria and leuconostocs were present after 7 days storage, with *Lactobacillus sake/curvatus* becoming the dominant organisms by day 14. The increase in the numbers of the rod-shaped lactic acid bacteria *vis-à-vis* cocci was even more noticeable in the highly acidified meat. After 14 days storage, the dominance of *Lact. sake/curvatus* was such that other microbial types were excluded.



a) Control (water addition)



b) Acidified



c) Highly acidified

Figure A3.5 **Lactic acid bacteria developing on minced beef rump with addition of acetic acid or a water control and stored at 5 °C**

DISCUSSION

The addition of acetic acid had a pronounced effect on the microbial population on the vacuum packaged minced rump of beef. Another study with lactic acid addition to minced rump beef showed similar trends (Witt, 1993). In both cases the relative sensitivity of the organisms to acids was *Brochothrix thermosphacta* > Enterobacteriaceae > pseudomonads > lactic acid bacteria. The pseudomonads were less sensitive to the acetic acid than the LAB during the first week of storage. These organisms are known to increase the pH of a meat juice medium - from < pH 6.0 initially to > pH 6.9 after 5 days growth (Drosinos, 1994). This production of alkali may buffer the environment in the locality of the cell and diminish the effect of the acid. An increase in numbers of *Pseudomonas* spp. was noted during the second week of storage of the highly acidified meat. This may have been due to a leak in the pack making oxygen available and reducing the concentration of CO_2. The synergistic action of acetic acid and CO_2 would be diminished and may allow the growth of the pseudomonads. The difference in cell numbers is greater than one log cycle. It is unlikely, therefore, to be caused by erroneous results. Yeasts were inhibited by acetic acid but their rates of growth were enhanced in the meat mildly acidified with lactic acid (Witt, 1993).

Acetic acid was generally more inhibitory than lactic acid, a feature noted by other workers (Gill and Newton, 1982; Adams and Hall, 1988; Young and Foegeding, 1993). Greer and Dilts (1993) in a study with various meat pathogens and spoilage bacteria found only marginal differences, dependent on the inoculated bacteria, between the two acids. The lactic acid bacteria surviving in meats acidified with acetic acid were identified in the present study but not in any of those noted above. It would appear that *Lact. sake/curvatus* is better adapted to survival in the acidified meat than leuconostocs and, to a lesser extent, *Carn. divergens*. Lactobacilli have been shown previously to withstand a lower cytoplasmic pH than other lactic acid bacteria (Kashket, 1987; Nannen and Hutkins, 1991). The effect of acids and low pH on the microbial associations developing on meats has been discussed (Chapter 4 and Chapter 7). Thus, from a commercial viewpoint, the use of acid rinses and sprays may tend to favour the growth of *Lact. sake/curvatus* on stored meat. This may be a disadvantage in MAP meats. The LAB dominate in this environment and the souring associated with large numbers of these organisms may be premature if acid sprays/washes are used.

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